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THE EFFECT OF CYCLOPHOSPHAMIDE (CYTOXAN) ON THE BLADDER MUCOSA

A Thesis

Presented in Partial Fulfillment of the Requirements
for the Degree Master of Science

by

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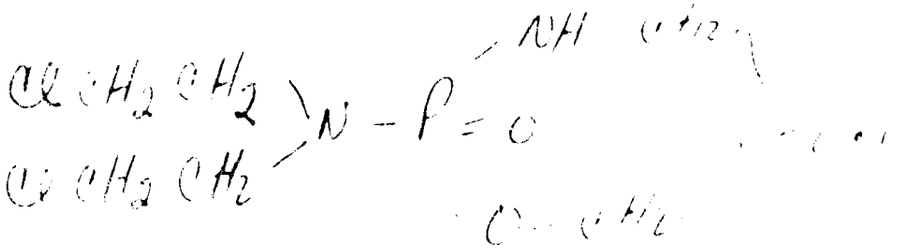
INTRODUCTION

Cytoxan brand of cyclophosphamide is a potent antitumor agent of the nitrogen mustard group which has been used in the treatment of patients with many kinds of neoplastic disease. Investigators have shown that Cytoxan is especially valuable in the treatment of patients with malignancies arising from hematopoietic tissues, malignant lymphomas, leukemia, some carcinomas arising from the breast and ovary and undifferentiated neoplasms. One of the reported effects of this agent is a chemical cystitis in a few cases (1-4%) during and following treatment with cyclophosphamide, (Mead Johnson Bulletin, 1964). It will be the purpose of this thesis to specifically investigate the cytologic effects of a single intraperitoneal injection of cyclophosphamide in rat bladder epithelium through studies conducted at the light and electron microscopic levels.

REVIEW OF THE LITERATURE

Nature of the Agent: Cytoxan, N, N-Bis (B-chloroethyl) N', O-propylene phosphoric acid ester diamide; Endoxan, B-518, cyclophosphamide) with the structure:



was synthesized by Arnold and Bousseaux (1958) in order to incorporate an antitumor radical in an inactive form that would be activated biochemically in the body. Initial experiments conducted by Arnold and Bousseaux (1958) utilizing tumor cells incubated at 37°C and at a concentration of 10^{-3} grams per milliliter of cyclophosphamide, established the relative inertness of the agent _in vitro_, while _in vivo_ experiments in fully developed rat tumors such as the solid Yoshida sarcoma, Walker-256 carcinoma and Jensen sarcoma, concluded in findings indicating that the apparent therapeutic index of cyclophosphamide was more than twice that of N-oxide mustard and ten times that of the ethylenimines, (Arnold and Bousseaux, 1958).

A study of one alkylating agent, notably cyclophosphamide, must include some overlap of information pertinent to other antitumor alkylating agents. Generally there are five major types of alkylating agents considered to be cytotoxic: mustards, ethylenimines, sulfonic esters, epoxides and certain N-alkyl-N-nitroso derivatives. The term

"mustard" generally refers to compounds containing one B-chloroethyl group attached to either a sulfur or a nitrogen atom, (Wheeler, G. P., 1962). Interest in the mustard compounds were initiated during WW I and again during WW II with the objective being the development of offensive anti-personnel weapons. The mechanism of action was recognized as cytologic, in that the opinions at that time were that the effect on hematopoietic and gastrointestinal tissues were due to a release of hydrochloric acid intracellularly with subsequent cytotoxicity and electrolyte fluid imbalance. Following WW II, there was a renewed interest in a series of nitrogenous analogues of the mustard gases, namely bis and tris -chloroethyl-amines. These cytotoxic effects were unlike anything observed to that time and resembled in many ways the cytologic manifestations following X-rays (Gellman and Phillips, 1946).

Cyclophosphamide is composed of nitrogen mustard linked by way of the nitrogen atom to a cyclic phosphoric-acid-esteramidase which masks the action of the mustard concluding in the absence of cytotoxic effects in vitro (Healy, J.B., 1964). The physical properties of cyclophosphamide are that it is stable at room temperature, soluble up to 4% in water and a 1% solution has a pH of 4.58. The source of the compound is Asta-Werke, A.G., Brockwede, Germany and the Mead Johnson and Company, (White, P., 1959). Cyclophosphamide, then, is an antineoplastic inactive cyclic phosphamide ester which was synthesized in an effort to incorporate an effective chemotherapeutic radical

which upon biological degradation would have a cytotoxic effect on tumoral tissue with a minimum of normal cell cytotoxicity (Dee Pres, et al., 1960, Haar, et al., 1960).

Mechanism of Action

Generally, the effect of the nitrogen and sulfur mustards are in cells which have an increased rate of proliferation. Such cellular susceptibility which can terminate in the death of the cells is manifest within the formed elements of the blood and the mucosa of the gastrointestinal tract. The effects on these cells by the mustard compounds reflects severe lymphopenia, granulocytopenia, thrombocytopenia, moderate anemia, vomiting, diarrhea, nausea, and anorexia. Histologic and cytologic examinations of the gastrointestinal mucosa, for example, demonstrates a progression from vacuolization and nuclear swelling of the epithelial cells to necrosis, desquamation, and hemorrhage. Lymphoid involvement is uniformly present throughout the body with lymphatic fragmentation evident within ten days following exposure. In the bone marrow early cytologic changes are swelling of the hemopoietic cells, alteration of their staining reaction and disappearance of mitotic activity leading to anaplasia. In vitro and in vivo studies have shown that mustard compounds inhibit oxygen consumption and glycolysis to varying degrees (Gilman and Phillips, 1946). The general mechanism of action of the mustards, including cyclophosphamide however, is not definitely understood (Freidman, O.M., 1963).

Cyclophosphamide was developed in order to produce a nitrogen

mustard derivative that while inert *in vitro* would theoretically be activated *in vivo* through phosphamidase activity in neoplastic cells (Journal of American Medical Assoc. -New Drugs and Therapeutics, Jan. 6, 1962). The biologic activity as studied through radio-isotope labeled cyclophosphamide indicates that cyclophosphamide breakdown occure in tumor tissue. It is hoped clinically that this agent will be transported in its inert form through the body and be absorbed and activated by metabolising malignant cells (Healy, J.B., 1964). The attachment of the phosphoryl group to a nitrogen mustard reduces the activity of the mustard compound by diminishing the basic properties of the central nitrogen atom thereby decreasing the ionizability of the chlorine atoms in the chloroethyl groups. To activate cyclophosphamide it is assumed that phosphamidases or phosphatases are theoretically needed to break the cycle group either at the phosphorus-oxygen or phosphorus-nitrogen bond liberating the mustard derivative. Since phosphamidases have been demonstrated in cancerous tissues (Gomori, 1948) and phosphatases in white blood cells, the cytotoxic effect on neoplasms and white blood cells can be explained (Coggins, P.R., *et al.*, 1960). To sum up then, the breakdown of the cyclic form to the active mustard by enzymes like phosphamidase permits the chlorine radicals in the chlorethyl groups to exert an alkylating activity (Des Pres, J.D., *et al.*, 1960). Phillips and Sternberg, *et al.*, in 1964 showed that in rats treated with cyclophosphamide, a circulating cytotoxic intermediaty was formed which was neither cyclophosphamide, or nor-nitrogen mustard. These determinations were based on the

calculation of the alkylating activity of the urine by the quaternization of the reagent, γ-(4-nitrobenzyl)-pyridine (NBP) and the extractibility of cyclophosphamide and nor-nitrogen mustard in chloroform. From these experiments it was concluded that there was alkylating activity (increase NBP reactivity) in rat urine following cyclophosphamide administration. In addition, 60% of the NBP reactivity was insoluble in chloroform and therefore the reactive substances were other than nor-nitrogen mustard and/or cyclophosphamide. The determination of increased urinary organic phosphate at 2 and 3 hours post cyclophosphamide injection which paralleled the NBP or alkylating activity suggested an in vivo metabolite of cyclophosphamide. McKenna, J.M., et al., 1962, through chlorimetric determinations to assay the presence of chloroethyl groups, in vitro, concluded that the cytotoxicity of nitrogen mustard seems to be directly related to the concentration of intact chloroethyl groups. The cytotoxicity of cyclophosphamide, however, was thought to be through the release of nor-nitrogen mustard and/or its enzymatic hydrolysis to cytoxyl alcohol or cytoxyl amine. Cytoxyl alcohol was described by Friedman, et al., in 1960 as a possible hydrolytic derivative of cyclophosphamide and proven cytotoxic on leukemic cells in vitro.

In cyclophosphamide treated mice, a collection of serum samples following intraperitoneal injections of 500 mg/kg exhibited marked inhibitory effects in mammalian cells (cell lines from L 1210 leukemia) within 15-30 minutes. A 2% treated serum resulted in 50% inhibition

of protein synthesis as compared to normal control animals. The inhibitory effect of the sera disappeared within a relatively short period of time. There was no inhibitory effect in cell lines treated with cyclophosphamide *in vitro*. In extracts from liver prepared from cyclophosphamide treated animals there was marked inhibitory effects on the protein synthesis of leukemic cells *in vitro*, but only a trace activity was demonstrated with the kidney and spleen extracts. There was no evidence of inhibitory activity when homogenates of neoplastic and normal mouse tissue were incubated with cyclophosphamide *in vitro*. There was some inhibitory effect, however, with liver homogenates incubated *in vitro* with cyclophosphamide. In addition to the marked inhibition of protein synthesis, there were marked morphologic cellular changes in the treated cell lines consisting of multinucleated giant cells with reduced amounts of cytoplasm suggestive of metaphase arrest. These experiments suggest the possibility that activation of cyclophosphamide does not occur to any appreciable extent in rat blood or in neoplastic tissue, but in the liver. Therefore, alteration of the drug to its active form probably does occur in areas other than in tumoral tissue through intermediary substances that may or may not be altered by the neoplastic tissue (Foley, G.E., *et al.*, 1961).

Animal experiments utilizing S^{35} labeled methionine in cyclophosphamide sensitive and cyclophosphamide resistant cell lines of the L 1210 leukemia show that cyclophosphamide significantly inhibited the incorporation of methionine in the sensitive tumor, while failing

to inhibit this amino acid in the resistant tumor. It is interesting to note that in these experiments there was no inhibition of methionine incorporation in either the sensitive or resistant tumor animals in the liver (Strozier, V.N., 1962). Therefore, the question and significance as to the role of the liver in the in vivo activation of cyclophosphamide, seems to be unsolved.

It has been mentioned previously that the nitrogen mustards inhibited oxygen consumption and cell glycolysis in vitro and in vivo. The effect of cyclophosphamide was studied in vivo on the following different mouse tumors, Ehrlech, Krebs 2, 1210 cyclophosphamide sensitive ascites cells, and S-91 melanoma. It was determined that inhibitions of anaerobic glycolysis and respiration occurred in vitro in tissue slices four hours after intraperitoneal injections of 200 mg/kg of cyclophosphamide. Seventy-two hours following injection both the aerobic and anaerobic glycolysis of the L 1210 cyclophosphamide susceptible cell line had been totally inhibited (Wright, K., 1960).

The mustard compounds have been shown to interfere with the mitotic divisions in a very wide variety of biological species. It is believed that the alkylating effect of the drug influences the mutagenic frequency of the genes. This alteration of the nucleic acids might result from interference with the synthesis and metabolism of the nucleic acids or from the alkylation of these acids. Influenza virus has been shown to be rendered non-infectious for mice by an in vitro exposure to increased concentrations of nitrogen mustard. Therefore, such deactivation might indicate the possibility that the

effect of the mustard is by way of direct alkylation of the nucleic acid rather than from influence upon some component system involved in nucleic acid synthesis (Wheeler, G.P., 1962). The incorporation of adenine -8-C^{14} into the DNA adenine of cyclophosphamide sensitive mouse tumors was shown to be inhibited by cyclophosphamide. There was no inhibition in the incorporation of the RNA-adenine. Chromatographic and radioautographic studies of tumor tissue extracts indicated that de novo synthesis of purine nucleotides was inhibited but there was little effect on the formation of nucleotides from the already formed adenine C^{14} (Wheeler, G.P., 1962).

Intradermal injections of DNCB (1-Chloro, 2-4 dinitrobenzene) were made in guinea pigs pretreated with cyclophosphamide in order to determine whether or not this drug blocked or prevented delayed hypersensitivity to DNCB. The results indicate that cyclophosphamide pretreatment delayed the appearance of contact dermatitis to DNCB suggesting that cyclophosphamide had a marked effect upon nuclear protein (Maguire, H.C., 1961).

The majority of the biochemical effects of the cytotoxic alkylating agents, and in particular cyclophosphamide, might be explained as direct or indirect results of nucleoprotein alkylation. The mutagenic or antimitotic effects would show involvement of the nucleus with alteration of the rate and production of DNA. The modified DNA could then alter the structure and formation of the messenger RNA which in turn affects the role and extent of protein synthesis (Wheeler, G.P., 1962).

Antineoplastic Effects of Cyclophosphamide

Cyclophosphamide chemotherapy for cancer has been used with varying degrees of success in many types of neoplasms. A review of the literature indicates that cyclophosphamide, like nitrogen mustard, is most active against neoplasms of the reticuloendothelial and hematopoietic systems. The following is the clinical evaluation of the drug listed by the type of neoplasm selectively described in the literature from the years 1960-1964.

Leukemia: In a study of forty-four children with advanced leukemia Fernbach, D.J., et al., 1962, showed that 18% of these children had complete remission, 11% had partial remissions and 23% had just hematologic remissions following cyclophosphamide chemotherapy. In a study of fifty children with acute leukemia, most of whom were resistant to 6-mercaptopurine, methotrexate and steroids, there were resultant remissions in 35% of the total cases, in which half of this total were partial remissions (Tan, C., et al., 1961). In studies of patients with chronic lymphatic leukemia Solomon, et al., 1963, described significant benefit in 44% of a total of 32 patients while 19% of these patients received only slight benefit following treatment with this oncolytic agent. A similar report of the efficacy of cyclophosphamide is noted in a study by Rundles, et al., 1962, in 28 patients with chronic lymphocytic leukemia in which a "fair to good response" was achieved in 40% of these patients following cyclophosphamide treatment. In a study of sixteen children with acute stem cell leukemia treated with cyclophosphamide there developed partial remis-

sions of from two to nine months in six of these patients (Sweeney, A., et al., 1963).

Hodgkin's disease, Lymphosarcoma and other Miscellaneous Hematopoietic diseases: Cyclophosphamide was shown to be "unusually" responsive in 33% of the cases of malignant lymphoma of Central Africa with remission rates of from four months to three years (Burkitt, D., Journal of American Medical Assoc.; Medical News, 1964). In a series of 31 patients with malignant neoplasms, three patients with lymphosarcoma had striking remissions (Papac, et al., 1960). In 253 cases of various malignant neoplasms treated with cyclophosphamide, there was "marked improvement with considerable transient objective change" in 46% of the cases, with two lymphogranulomatoses and reticulum cell sarcomas showing complete remissions, (Gerhartz, H., et al., 1960). The best results in 31 patients with various neoplasms treated with cyclophosphamide were in three patients with lymphosarcoma who had striking remissions (Papac, et al., 1960). There was significant responses in three out of five children with Ewings sarcoma treated with cyclophosphamide (Sutow, et al., 1962). Twelve patients out of twelve with lymphoma achieved some degree of remission following cyclophosphamide therapy, but the remissions were of a short duration with the exception of two cases of Hodgkin's disease who acquired a remission of more than one year (Sweeney, A., Tuttle, J., 1963). Some degree of actual remission was achieved in 11 patients with Hodgkin's disease, especially in those patients in whom this was the initial

treatment for the disease, (Olmer, et al., 1962). The most "satisfying results" following administration of cyclophosphamide to 49 patients with malignant disease were seen in seven patients with Hodgkin's disease. One patient with a γ-plasmacytoma had a two year remission (Fritzche, et al., 1962).

In nineteen patients with Hodgkin's disease there were six patients with significant benefits following treatment with cyclophosphamide of periods ranging from 1½ to 15 months. There was a slight improvement on seven patients with the remaining six patients considered failures or unevaluable. In a total of eight patients with lymphosarcoma, there were four patients who received significant benefit and one patient who received a slight benefit following cyclophosphamide chemotherapy (Solomon, J., Alexander, M., & Steinfeld, 1963). A comparison between mechloroethamine in 98 cases of Hodgkin's disease and lymphosarcoma groups indicated that there was a 60% favorable response in those groups of patients treated with cyclophosphamide, 55% in those treated with uracil mustard and 25% with mechloroethamine (Gold, et al., 1962).

In a study comparing the effectiveness of cyclophosphamide and nitrogen mustard, Zubrin, et al., 1961, determined that "good to excellent objective responses" were achieved in the following diseases: Hodgkin's disease eight out of nine with nitrogen mustard, and 34 out of 72 with cyclophosphamide; in malignant melanoma there were no cases in his study that responded to nitrogen mustard, while 2 out of 8 cases responded to cyclophosphamide therapy. In multiple myeloma, no

cases responded to nitrogen mustard, while 4 out of 30 responded after cyclophosphamide chemotherapy. Cyclophosphamide seemed to be "unusually" effective in suppressing the growth of more primitive neoplastic lymphoid elements (Rundles, R., et al., 1962). Treatment of eight patients with neuroblastoma with cyclophosphamide resulted in three patients experiencing relief of symptoms and transient decrease of the tumor. In seven children with rhabdomyosarcoma, there were three patients who obtained temporary objective benefit with cyclophosphamide. In four children with osteogenic sarcoma there was one patient in which there was a regression of metastisising tumor with this chemotherapeutic agent. "This might suggest cyclophosphamide may affect some tumors not responsive to other alkylating agents and thus it may have a truly different antitumor spectrum." (Pinkel, D., 1962). In a total of seven patients with multiple myeloma treated with cyclophosphamide there was one patient with significant benefits, and one patient with slight to moderate degree of improvement (Solomon, J., Alexander, M., Steinfeld, J., 1963). In nine patients with myelomatoses there were no radiological or electrophoretic changes (Healy, J., 1964). In 29 patients receiving cyclophosphamide for multiple myeloma, objective evidence of a favorable effect was observed in six patients while there was subjective improvement in three other patients (Rivers, S.L., 1963).

An inhibitory effect by cyclophosphamide was determined in two patients with metastatic angiosarcoma which concluded in "striking" regression of their pulmonary metastasis after failing to respond to

a combination of the alkylating agent Theo-TEPA and the anti-metabolate Methotrexate. This suggested that the antitumor action of cyclophosphamide may be significantly different from that of the other known alkylating agents (Greenspon, 1961).

Miscellaneous Malignant Neoplasms and Disease Entities

In four out of five patients treated for metastising nasopharyngeal cancer there were remissions ranging from five to eight months following cyclophosphamide therapy. In one patient with cancer of the pancreas with hepatic metastasis, and one patient with an epigastric tumor with liver metastasis there were general improvements of condition from six months to a year, (Haddad, N., 1963). In a 55 year old female patient with recurrant epidermoid epithelioma of the upper maxillary region there was dramatic improvement after ten days of cyclophosphamide chemotherapy with the patient alive and well eighteen months later (Laroum, R., 1963). In eight patients with ovarian carcinomas treated post operatively with cyclophosphamide, five patients are alive 2½-4 years after treatment as compared with an 82% mortality post-operatively without chemotherapy (Brubl, R., 1962). In six out of ten cases of ovarian carcinoma there was distinct remissions of varying lengths (Healy, J., 1964).

Marked objective and subjective improvement was noted in one patient with an infiltrating duct carcinoma with mediastinal and pulmonary metastasis following cyclophosphamide therapy (Gonsales, E., 1961). Cyclophosphamide in combination with anti-metabolite or antibiotic antitumor agents have shown promise in the treatment of malig-

nancies of the stomach and hepatobiliary areas (Hurley, J., 1961). Twenty-nine patients have shown definite regression of their disease following cyclophosphamide chemotherapy including three with malignant melanoma, six with carcinoma of the ovary, nine with malignant lymphoma and three with adenocarcinoma of the breast (Coggins, P., Rawdin, R., Eisman, S., 1960). In 45 patients with a variety of histologically confirmed malignancies including 24 bronchiogenic and six gastric neoplasms there was radiologic and clinical improvement in 11 patients and subjective improvement in 22 patients (Hammer, et al., 1960). In one patient who had undergone orchiectomy and subsequent radiation therapy for a testicular carcinoma who had developed metastatic lung envolvement there was marked regression following cyclophosphamide chemotherapy (Galle, 1961).

Beneficial success was achieved in a patient with psoriasis of the scalp following treatment with cyclophosphamide for widespread carcinoma of the colon. The psoriasis has cleared and the patient has been in psoriasis remission for eighteen months. In a 17 year old female without malignant disease who was treated with cyclophosphamide for extensive psoriasis and psoriasis arthritis results indicated there was no skin or joint disease five weeks after therapy. In two out of three patients with mycosis fungoides there has been marked improvement after cyclophosphamide chemotherapy (Lasalo, et al., 1961). In three cases of Waldenstrom's Macroglobulinemia there was dramatic objective and subjective improvement with decrease in serum

macroglobulins up to the present time following treatment with cyclophosphamide (Bouroncle, et al., 1964; Bouroncle, 1965). In a total of two patients with Waldenstrom's Macroglobulinemia there was significant response of from six and eleven months following cyclophosphamide therapy (Solomon, J., Alexander, M., and Steinfeld, 1963).

It appears that the efficacy of cyclophosphamide chemotherapy is in those hematopoietic malignancies such as Hodgkin's disease, lymphosarcomas and leukemias, particularly chronic lymphatic leukemia. In addition, frequent responses are reported in those solid malignancies such as ovary and breast along with miscellaneous metastatic neoplasms. "It is important to realize that the best that can be expected at the present time from cyclophosphamide therapy is a remission of the neoplastic process which in many instances is only temporarily; one hopes to ease the symptoms and perhaps delay death for a while." (Healy, J., 1964). I have purposely neglected to include the dosage regimen in these listed pathologic conditions due to the wide fluctuations in initial and maintenance dosages as expressed in the patient management by the various author clinicians. I will briefly describe the biological effects of various maximum dosage regimens in the next section.

Preliminary studies in leukemic mice show that an antimitotic agent such as cyclophosphamide can be utilized effectively in bone marrow transplants by enhancing the antileukemic effects through reductions in the mitotic rate of tumor cells and, in addition, decrease

the incidence of secondary disease through interference in the immunologically competant cells from the bone marrow transplant (Mathe, G., et al., 1962). Cyclophosphamide administered to guinea pigs sensitized to egg albumin prevented the manifestation of an anaphylaxis reaction indicating that "cyclophosphamide provides a profound and specific depression of the nucleic acid metabolism of the antibody forming machinery." (Maguire, H., et al., 1961). The effect of cytotoxic agents in terms of the immune response seems to be an inhibition of the "induction phase" of antibody formation through strong inhibition of nucleic acid synthesis particularly deoxyribonucleic acid (Berenbaum, M., 1960). The role of cyclophosphamide in wound healing as conducted in animal experiments indicates that it retards the exudative inflammatory process and formation of granulation tissue. Proliferating epithelium in granulating wounds appears relatively immature as evidenced by large hyperchromatic nuclei and abundant cytoplasm associated with pseudoepitheliomatous hyperplasia (Des Pres, J., 1960).

Biological and Toxicologic Effects

Three distinct factors seem to influence the biological effect of cyclophosphamide; the nature of the subject treated, i.e. whether it be man or different experimental animals; the dosage of the agent complexed with the intervals of administration; and the route of injection.

Early studies of the biologic and toxicologic effects of cyclophosphamide in animals revealed leukopenia, weight loss and subsequent death depending on the amount and duration of the agent given to these experimental animals (Des Pres, et al., 1960).

In mice and rats the oral LD_{50} values were 350 and 94 mg/kg respectively. Rats survived repeated oral dosages of 6 mg/kg for six weeks whereas dogs survived with a lesser daily oral dose of 5 mg/kg for six weeks. The pathologic manifestations in these animals at this regimen consisted of splenic congestion, depressive reaction of the hematopoietic tissues including the bone marrow. Following single sub-lethal dosages, the peripheral blood exhibited leucocyte depression with recovery in three weeks and erythroid depression followed by complete recovery in twelve days (Wheeler, A., Danaby, D., et al., 1961). The major manifestation of toxicity in experiments with rats and mice were weight loss, ruffled hair, diarrhea and hematopoietic depression and death in periods of time ranging from 1-10 days (Love, M., 1959). The overt acute manifestations of toxicity in mice after cyclophosphamide therapy are similar to those seen in mice following nitrogen mustard administration. Injections of 600-700 mg/kg of cyclophosphamide in mice elicited terminal convulsions which seemed to be related in time to respiratory paralysis probably as a result of anoxemia. Changing the regimen from daily injection to weekly injections, increased the median survival rate of these animals (Love, M., 1959). In studies conducted on rats and dogs comparing the efficacy of cyclophosphamide on various neoplasms as a function of dose interval, routes of administration and sex, it was concluded that no significant sex differences occurred in terms of neoplastic effect or toxic manifestations; in rats the oral route was less toxic than the intravenous route; in dogs a single acute dose or

a 48 hour infusion was tolerated on a biweekly regimen, but only one half of the dose was tolerated if administered daily or weekly. "The longer the delay between dosages, the longer the delay was between onset of leukopenia and death." Toxic signs in these animal experiments included emesis, diarrhea, proteinurea, and hematuria. Thrombocytopenia was not severe, although leukopenia and anemia occurred regularly (Fallotta, A., et al., 1960). Cyclophosphamide in dosages ranging from 37.5 mg/kg to 600 mg/kg in mice over a five day period produced more specific damage to the lymphatic areas at lower doses than nitrogen mustard or triethylinemolamine (Scaltrini, G., et al., 1962). Treatment below the minimum tolerable dose in mice (200 mg/kg I.V. or 40 mg/kg for seven days i.p.) and rats (120 mg/kg I.V. or 20 mg/kg for seven days i.p.) indicates that the granulocytes recovered faster than the lymphocytes. Significant pathologic change was noted in the intestinal tract, bone marrow, and lymphoid tissue but not in the thymus in animals dying after treatment with cyclophosphamide. Cyclophosphamide when injected into the albumin of fertilized chicken eggs induced generally atrophic development of the chicken embryo. This was manifest by micromelia of the legs, parrot beak, absence of eyelids, and visceral and cardiac lesions (Gerlinger, P., et al., 1963).

Injections of single dosages of from 25-60 mg/kg for five days of cyclophosphamide in dogs intrathecally showed no clinical evidence of toxicity in the lower dosages but gastrointestinal bleeding leading to death of the animals, occurred in the 50-60 mg. dosages. In all

dosage regimens there was no central nervous system disorders at necropsy (Bland, J., et al., 1961). Cyclophosphamide, 3.2 mg/kg, injected intra-muscularly in leukemic mice resulted in no immediate cytologic changes. Mitosis of the leukemic cells which were in progress at the time of injection, or started a short time following injections, were little affected. In several hours, however, the mitotic index dropped indicating that the number of cells entering mitosis was decreased. This might suggest a delay in the premitotic process. After 24 hours the mitotic index returned to its initial value. The mitosis themselves, however, were abnormal and progressed slowly. Some of the mitotic configurations seemed to be aborative or produced indivisible daughter cells leading to a decrease in cell population. The cell injury probably occurred during interphase, many hours before mitosis and this might indicate that tumor cells were the most sensitive to the chemical action of cyclophosphamide at this time. It is interesting to note that within 48 hours the regular mitotic figures again became evident carrying the same chromosome complement as the tumor cells before therapy, and indicating that some of the cells were barely affected (Kovacs, S., et al., 1960).

"When treating malignant diseases with cytotoxic agents, we want to use dosages as high as possible to get the best possible effect in tumor tissue: but not so high that the damage to important normal tissues becomes dangerous or irreversible. The most serious side effect of the cytotoxic agents is damage to the bone marrow cells." (Nissan-Meyer, R., et al., 1960). The major toxicologic side effect

of cyclophosphamide in the treatment of tumor neoplasms are as follows:

Disturbances in the hematopoietic tissue: Cyclophosphamide administered to 59 children with neoplastic disease in which leukopenia was deliberately induced through dosages of 150-200 mg per square meter initially, and maintenance dose individually altered to maintain a peripheral white count of from 2,000 and 5,000 per cubi-millimeter, was manifest by moderate relative granulocytopenia and monocytosis with an occasional case in which increases in eosinophils was noted. In addition, there was moderate anemia with hemoglobin of 7-9 grams in many patients who had received the drug for periods of up to two years. There was relative hypoplasia, mild granulocytopenia, maturation arrest of moderate degree in both the granulocytic and erychrocytic elements of the bone marrow. All hematologic depressions returned to their initial levels after termination of therapy, (Sweeney, M., Tuttle, A., Ettaldorf, J., et al., 1962). In 44 children who had received 2.5 mg/kg - 5 mg/kg for 5-10 days initially followed by a maintenance dose culminating in total dosage ranges of 374-470 mg/kg total, there were 93% of the patients exhibiting a decrease in total white blood count, ~75% of whom had less than 1500 cells per cubic millimeter (Fernbach, D., et al., 1962).

In a study in which eleven out of twenty-four patients with Hodgkin's disease received favorable results or actual remissions, it was determined that cyclophosphamide was less damaging to the erythrocytes and thrombocytes than other chemotherapeutic agents (Olmer, et al., 1962). Following chemotherapy with cyclophosphamide

in 44 children with advanced leukemia it was observed that the most common side effect manifest was leukopenia with an absence of thrombocytopenia and anemia. It is of interest to note that the leukopenia resulting from cyclophosphamide chemotherapy in this study developed more rapidly in a higher dosage regimen with the lowest white count occurring between 5 and 21 days from initiation of therapy. Total cumulative dose did not correlate, however, with the degree of leukopenia. It was further noted that leukemic involvement of the central nervous system was not inhibited by cyclophosphamide chemotherapy (Fernbach, D., et al., 1962).

In a study comparing the anti-neoplastic effect of cyclophosphamide in a massive dosage regimen (45-80 mg/kg I.V. at 4 week intervals) versus a fractionated initial regimen followed by daily maintenance dosages (7.5 mg/kg for 4-6 days followed by 50-150 mg orally) in 33 patients with metastatic breast or ovarian cancer, it was concluded that no superiority could be demonstrated as "induced marrow toxicity had no effect on the rate of remission." However, in this study, there was an earlier increase in toxic mortality following the larger massive drug injection regimen, (Coggins, P., Bisman S., et al. 1961).

In a study of 105 records involving individuals under cyclophosphamide chemotherapy there were 24 patients in which the white blood count went below 2,000 cells per cubic millimeter, 5 patients in which the count was below 1,000 cells per cubic millimeter and 46 patients whose count was below 3,000 cells. There was no corre-

lation between leukopenia and degree of response (Healy, J., 1964). The maximum safe dosage initially in patients without hematopoietic disease seems to be 10-20 mg/kg to a total of 40 mg/kg followed by a maintenance dose commensurate with a white count of 2-5,000 cells per cubic millimeter (Rundles, et al., 1962). The primary advantage in the use of cyclophosphamide seems to be the lesser destructive effect on the megakaryocytes and thrombocytes for the equivalent of the same leukopenic effects of other alkylating agents. In addition, there is the distinct advantage in its in vitro inactivity, as compared to nitrogen mustard, in that there is no reaction at the site of injection, coupled with the fact that one is not limited to intravenous routes as cyclophosphamide may be administered per os, intramuscularly, intra-peritoneally, or in the pleural or pericardial sacs (Solomon, J., Alexander, M., Steinfeld, J., 1963). In a comparison of the effect of cyclophosphamide and nitrogen mustard in terms of toxic side effects in 14 leukemic patients it is concluded that the cyclophosphamide attacks a generation of marrow cells about one week older than the generation of marrow cells attacked by nitrogen mustard. This could be explained on the basis of a lack of specific enzymes in the immature or younger cells. In addition, the megakaryocyte series is less vulnerable to cyclophosphamide than those cells in the granulocytic series, again explained on the basis of the lack of enzymes necessary for activation of the agent (Nissen-Meyer, R., et al., 1960). The effectiveness of cyclophosphamide chemotherapy seems to be enhanced to some degree by the intravenous routes of

administration (Haar, H., et al., 1960), but at the same time patients seem to be less tolerant to the side effects through this route of administration (Hammer, O., et al., 1960).

Alopecia: Cyclophosphamide induced alopecia has been one of the unique side effects of this chemotherapeutic agent. Evaluations of 44 children with advanced leukemia indicated that the second most common side effect in cyclophosphamide chemotherapy was alopecia of the scalp, brow, eyebrows, and eyelashes (Fernbach, D., et al., 1962). In a study of 25 individuals with various malignant neoplasms who were undergoing cyclophosphamide chemotherapy, ten developed partial to complete alopecia in time intervals ranging from 2-50 weeks from initiation of therapy. There was no correlation between the incidences or severity of alopecia and the total dose or on the degree of leukopenia. The alopecia regressed after cessation of treatment (Falkson, G., et al., 1963). A study in the hair cycle in six patients ages 4-61 receiving from 50-300 mg/day of cyclophosphamide for various malignancies indicated that the age and clinical condition of the patients did not seem to play any significant role in the severity or frequency of the alopecia. In this study it was felt that the hair at the periphery was not as likely to fall out than hair in a central area. Histiologically, there was no explanation as far as the hairs themselves were concerned. It is possible that the alopecia is a manifestation of a metabolic disturbance of the mitotic activity of the hair matrix cells (Brown-Falco, O., 1961). The hair root is one of the most rapidly proliferating tissues of the body, ranking with the cells of the gastrointestinal tract and bone marrow, and

since cyclophosphamide has seemingly a more specific effect on proliferating cells, the alopecia might result from an interruption of the mitotic activity of these rapidly proliferating cells (Gold, G., et al., 1962).

Gastrointestinal disturbances: Disturbances of the gastrointestinal tract compose another area of the body in which toxic manifestations of cyclophosphamide activity is evident. Ten out of forty-five patients with inoperable cancer treated with cyclophosphamide complained of fatigue, and anorexia; ten patients suffered episodes of nausea and vomiting which was controlled by antiemetics (Hammer, O., et al., 1960). Cyclophosphamide employed over a three year period to 59 children with neoplastic disease resulted in gastrointestinal distress varying from mild anorexia to severe vomiting in almost all of the children whose dose range was high in the initial phase of treatment i.e. 150-200 mg./square meter per day intravenously. The most severe complaints, however, tended to subside in several days or could be controlled (Sweeney, M., Tuttle, A., et al., 1962, 1963). In studies comparing cyclophosphamide with mannitol mustard in 32 patients with various malignant diseases, it was observed that both agents induced nausea and vomiting which was directly related to dose. With cyclophosphamide given per os, no instances of nausea and vomiting occurred (Papac, R., et al., 1960). In 16 patients with advanced adenocarcinoma of the gastrointestinal tract treated with cyclophosphamide and Actinomycin D, there was evidence of more severe toxicity greater than what would normally be expected using either

drug alone, as evidenced by the fact that in 88% of the cases nausea and vomiting occurred, followed by 12% of the cases who developed gastrointestinal hemorrhages (Moertl, C.G., et al., 1963). It is of interest to compare nitrogen mustard and cyclophosphamide in terms of the manifestation of achlorhydria found after gastric irradiation. It was demonstrated that nitrogen mustard causes a transient achlorhydria probably due to action upon cell mitosis with decline of cell reproduction and cell function. On the other hand, cyclophosphamide did not produce achlorhydria supposedly because the postulated enzyme phosphamide necessary for the activation of cyclophosphamide does not exist in great quantities in the stomach (Baume, P., 1962).

Other miscellaneous symptoms attributed as side effects in cyclophosphamide therapy has been hematuria and chemical cystitis (to be discussed in the next section), redness and ulceration of the buccal mucosa and skin eruptions, (Tan, C., et al., 1961), and jaundice with accompaning abnormal liver function studies in two leukemic children under chemotherapy (Fernbach, D., et al., 1962). In one case of initial and maintenance treatment with cyclophosphamide in a pregnant woman with Hodgkin's disease there was evidence of teratogenic effects on the child by this chemotherapeutic agent. After spontaneous onset of labor, a four pound, four ounce boy was delivered with four toes on each foot, a flattened nasal ridge, a slightly hypoplastic middle phalanx of the fifth finger and bilateral inguinal hernia sacs. Chromosomal analysis on peripheral blood leukocyte cultures revealed

a total of 46 chromosomes with normal karyotype. If cyclophosphamide exerts its effect by alkylation of the susceptible radicals in the nucleoproteins, cells with the greatest mitotic activity would be the most affected. The mitotic poison might cause malformations by acting as growth inhibitors on certain tissues in a particular phase of growth at any one particular time. The initial treatment in this woman with this agent coincided with the embryonic development of the particular tissues affected (Gruenberg, et al., 1964).

Finally, I think it of general interest to note that according to some published accounts, side effects induced by cyclophosphamide were either entirely absent or at best, extremely minimal. In the treatment of a 56 year old man with infiltrating duct carcinoma without metastasis treatment culminated in a total dose of 6000 mg (6 grams) in which there was radiographic improvement and absolutely no side effects (Gonzalez, E., 1961). In the treatment of ten children with various malignancies only six had any appreciable side effects and that was limited to a very transient leukopenia. Initial dosages were at the minimum of 35 mg/kg. None of the children showed evidence of nausea or vomiting (Cromblett, H., 1960). Cyclophosphamide, in dosages of 200-400 mg per day intravenously, in five patients with metastasising nasopharynegeal cancer showed side effects limited to spontaneously reversible leukopenia (Haddod, N. et al., 1963).

Hematuria and Sterile Cystitis Associated with Cyclophosphamide Chemotherapy: Another unique and sometimes fatal side effect in cyclophosphamide chemotherapy has been the infrequent occurrence of a sterile

cystitis in some patients. It is by no means a consistent side effect in the vast majority of the reported cases, but its occurrance in some of the cyclophosphamide managed patients had been severe enough to completely interfere with the management of the disease process and sometimes has actually contributed to the patients death. It is the generally held opinion by many chemotherapists familiar with cyclophosphamide treatment that the frequency of chemical cystitis might be proportional to the increased dosages of the drug. However, these opinions are at variance with at least one other report (Forni, A., et al., 1964) in which there was no correlation between the dose and severe cytologic atypias observed from patient urocytograms.

Moderate to severe hemorrhagic cystitis was seen in many patients in a study of 16 children under treatment with cyclophosphamide with acute stem cell leukemia. This cystitis seemed to coincide in those children who received long term continuous oral therapy (Sweeney, A., Tuttle, J., Ettoldorf, et al., 1963). Urinary frequency, dysuria and hematuria occurred in 20 out of 56 patients receiving cyclophosphamide for various malignant neoplasms. The symptoms themselves did not appear until 5-6 weeks after courses of continuous therapy. The hematuria observed varied from microscopic to gross bleeding. Urine cultures and colony counts were normal. In the few cases where thrombocytopenia occurred, the hematuria that developed was more severe and moreprolonged. Cystoscopic examination accomplished in two children who did not have thrombocytopenia revealed numerous hemorrhagic, vesicular, and ulcerative mucosal bladder lesions. Biopsy of

these lesions indicated no neoplastic cells. It was noticed by cystographic and cystoscopic examinations as well as at autopsy that these lesions tended to heal with considerable scar tissue. In patients with severe involvement, bladder contracture occurred, and the duration of these bladder symptoms varied from a few hours to weeks at a time with very common recurrances (Sweeney, M., Tuttle, A., Etteldorf, J., et al., 1962). Hemorrhagic cystitis was attributed to four cases of leukemic children undergoing cyclophosphamide chemotherapy by Fernbach, et al., 1962. This cystitis started with dysuria followed by hematuria. In one child there were recurrant episodes that ceased when therapy was discontinued. In another child the hematuria and dysuria persisted 17 days following cessation of therapy. One other patient in whom chemotherapy was continued during the manifestation of these symptoms had complete disappearance of the symptoms after 34 days spontaneously. All patients received over 300 mg/kg cumulated dose of cyclophosphamide before symptoms appeared. In a study of seven women with various malignant tumors treated with cyclophosphamide in cumulative dosages ranging from 2.7 grams to 79.4 grams there was hematuria attributed to bladder toxicity to the drug in all cases. Histologic damage of the bladder was found in all patients at necropsy with one woman dying of uncontrollable hematuria after 16 months of cyclophosphamide chemotherapy (Kaufmann, J., 1963). In one patient with lung neoplasm along with mediastinal cervical metastases who showed "frank regressions" of her pulmonary lesions following cyclophosphamide chemotherapy there developed during her treatment a hemorrhagic cystitis which was still present two months after treatment

(Casares, T., et al., 1961). In a three year old child treated with large intravenous infusions of cyclophosphamide for three days followed by maintenance dosages of 8 mg/kg per os for a metastatic neuroblastoma, there developed one month after therapy gross hematuria which progressed to "frank" bleeding requiring frequent blood transfusions which persisted for two months until his death. A suprapubic cystostomy by trochar was necessary to relieve bladder obstruction by blood clots and cystoscopy revealed multiple petechial hemorrhages of the bladder, neck and wall. There was no tumor in the bladder, cystograms were normal and repeated urine cultures were negative. At necropsy the bladder was large and its wall was thickened and appeared fibrous. Histologically there was marked vascularity of some areas of the epithelium and in the tissue below the epithelium. The muscular wall was considerably fibrosed extending to the serosal surface. Such pathology indicates that cyclophosphamide can cause a chronic fibrosing and hemorrhagic cystitis that is severe enough to produce urinary obstruction and "exsanguinating" hemorrhage (George, P., 1963). In 93 cases of various malignancies under therapy with cyclophosphamide there were two cases in which cystitis occurred. In one patient with lymphatic leukemia, bladder bleeding precipitated death (Healy, J., 1964). Dysuria followed by hematuria developed in four children with acute leukemia following cumulative dosage ranges of from 314-470 mg/kg of cyclophosphamide. Repeated urine cultures were negative (Fernbach, D., et al., 1962). Two out of five patients receiving daily maintenance dosages of 2.5 mg/kg of cyclophosphamide for Ewing's sarcoma

developed a sterile hemorrhagic cystitis (Sutow, W., et al., 1962). At dosages of 4 mg/kg per day in 29 patients receiving cyclophosphamide for multiple myeloma, two patients developed a "sterile" cystitis (Rivers, S., et al., 1963).

Phillips, F., et al., in 1961 conducted the most complete study of the relationships between cyclophosphamide and its "active" derivatives on the urinary bladder. In his studies on rats and dogs following dosage regimens of from 29-333 mg/kg of cyclophosphamide, it was determined that the urine volume was significantly decreased during the first two hours following intraperitoneal administration of 222 mg/kg of cyclophosphamide. The urine in these experimental animals contained a high concentration of alkylating substances which were neither cyclophosphamide or nor-nitrogen mustard. The LD_{50} in his experimental rats was determined to be 182 mg/kg. Most of the deaths occurred after the seventh day following a dosage of 222 or 333 mg/kg. Lymphopenia and neutropenia in these animals began within 24 hours and was extreme between 2 and 8 days. Sacrificed animals showed extensive ulcerations of the bladder mucosa with a bloody exudate in the lumen. This exudate contained cellular debris, fibrin and inflammatory cells. The ulceration remained prominent through the sixth day with repair and regeneration evident by the fourth day. At this time mitotic activity was noted in the epithelium outside the zones of ulceration along with fibroblasts, histiocytes and polymorphonuclear neutrophils in the submucosa. The bladder epithelium was intact by the eighth day. By the 12-13th day the epithelium was

normal but there were numerous histiocytes laden with hemosiderin throughout the submucosa. Edema which was evident after 24 hours was still present after 12-13 days. Evidence of renal lesions were found in some of the animals consisting of focal areas of hydronephrosis and necrosis of the convoluted tubules. There was evidence of edema within the prostate but it was thought to be due to contiguous damage from toxic urine. Dosages in rats as low as 29 mg/kg were toxic to the bladder and produced mucosal hemorrhages. Injections of "toxic urine" directly into the bladder of female dogs from donor animals treated with cyclophosphamide intravenously showed marked areas of hemorrhage on the luminal surface of the bladder. Widespread edema was also observed along with areas of hemorrhage within the submucosa. Some of the animals showed focal muscle necrosis. These pathologic changes were consistent in those seen in the donor animals treated intravenously with cyclophosphamide. Direct injections of cyclophosphamide into the bladder lumen of female dogs failed to show any significant pathology.

Intraperitoneal injections of nor-nitrogen mustard into these experimental animals failed to show any evidence of bladder damage. It is of interest to note that when cyclophosphamide was injected in low dosages along with the osmotic diuretic mannitol, complete protection of the bladder was observed. This was not the case with higher dosages due to an apparent anti-diuretic effect caused by these higher dosages (Phillips, et al., 1961). This experimental animal study indicates the following:

(1). Intraperitoneal and intravenous injections of cyclophosphamide into rats and dogs at dosages ranging from 29-333 mg/kg induce severe bladder toxicity.

(2). Evidence of higher concentrations of alkylating agents were found in the bladder following cyclophosphamide administration. However, the toxic substances were not cyclophosphamide per se or nor-nitrogen mustard. The toxicity of the bladder urine was proved through donor-recipient bladder urine transfusions.

(3). The bladder damage can be minimized through increased diuresis.[1]

(4). The site(s) of cyclophosphamide activation are uncertain as are the nature of some of the intermediary cytotoxic derivatives.

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[1]The importance of adequate diuresis is manifest in the low incidence of chemical, sterile cystitis resulting from cyclophosphamide therapy at University Hospital, Columbus, Ohio. In a private conversation with Dr. Bertha Bouroncle, Associate Professor of Medicine, University Hospital, she indicated that she was not aware of a single case of cyclophosphamide induced cystitis at the hospital for over a year and a half. Her explanation for this absence of cystitis was due solely to proper hydration of the patient. Failure to maintain adequate hydration became manifest in one patient suffering from Hodgkin's disease, who admitted of becoming somewhat "dehydrated" during final exams while under treatment with cyclophosphamide, and who developed symptoms of urgency and frequency of urination which was attributed to the cyclophosphamide. With proper hydration, the symptoms ceased.

My general conclusions in reference to this subject of cystitis resulting from cyclophosphamide following a review of literature are as follows:

(1). There seems to be a correlation between dose and frequency of occurrance but no correlation in the relative time of occurrance during or following the chemotherapy administration. The relative smaller percentages of clinical occurrances in the literature as compared to the other biologic and toxicologic manifestations of the drug is either due to proper hydration of the patients in the majority of individuals, or to a low susceptibility to metabolic products of cyclophosphamide.

(2). The cytotoxic products producing the pathological condition in the bladder is not limited to excreted unchanged form of cyclophosphamide or nor-nitrogen mustard.

(3). The activation of cyclophosphamide is most likely due to an enzymatic process occurring probably at multiple sites of the body. The enzyme most likely responsible is phosphamidase which transforms the "inactive" form of cyclophosphamide to the "active" form resulting in cytotoxic derivatives which can induce bladder damage.

METHODS AND MATERIALS

Experimental Animals for Electron Microscopy

Thirty-two white, male Westar rats were obtained on 6 January 1965 from the Carworth Farms, New City, New York. The weights of these animals on delivery ranged from 240-333 grams. The animals were placed in individual wire cages without bedding material in the air conditioned animal room of the Department of Pathology, Ohio State University, School of Medicine. The animals were given free access to water and Purina Laboratory Chow manufactured by the Ralston Purina Company. According to the manufacturer's specification as listed on the food bags, this food contains not less than 23% animal protein, not more than 6% crude fat fibre, not more than 9% ash, and not less than 4.5% crude fat. The constituents of the food consists of meat and bone meal, dried skim milk, germ meal, liver meal, dried beet pulp, dried extruded corn, oat [illegible]ngs, soybean meal, dehydrated alfalfa meal, cane molasses, animal fat preserved in butylhydroxy-anisole, vitamin B_{12}, calcium panthothenate, folic acid, riboflavin, brewer's dried yeast, thiamin, niacin, vitamins A, D, and E, activated plant sterol, phosphorous iodised salt, $FeNH_4$, citrate, $MnSO_4$, and ZnO. The animals were not given any other food supplement. The animals were observed daily for a period of nine days during which time there was additional weight gain in all animals and no obvious manifestation of disease. All animals were alert and responsive, the stools were hard formed and the urine was a clear yellow. On the evening prior to

the initiation of the experiment, 14 January 1965, each individual animal was again weighed with the weights of these animals ranging from 259-342 grams.

Six vials, each containing 500 mg of cyclophosphamide, was obtained from the pharmacy of the University Hospital, Ohio State University three days prior to beginning of the experiment. At approximately 0500 hours on the morning of 13 January 1965, twenty-five cubic centimeters of sterile distilled water was injected into each of the cyclophosphamide vials. The injections were made through a 25 cc sterile syringe and a series of six sterile 20 guage needles following the removal of the metal protective cap on the cyclophosphamide vial and subsequent cleansing of the rubber stopper with sterile cotton soaked in absolute alcohol. Introduction of 25cc's of sterile water into the vial is the recommended procedure for cyclophosphamide reconstitution (Cytoxan: Mead Johnson Laboratories, February, 1964). The solution was agitated and allowed to stand until it was clear. Upon complete reconstitution, the concentration of cyclophosphamide was calculated to be 20mg per lcc.

At approximately 0600 hours on the morning of the 15th of January each of the thirty animals was prepared for an intraperitoneal injection of cyclophosphamide. The quantity of the agent was determined by the individual weights of the animal at a dose of 222 mg/kg of animal in constant volume of approximately lcc per 100 grams of animal (Phillips, F., et al., 1961). The animals were held firmly

and the agent administered intraperitoneally with a series of sterile 5cc syringes and 25 guage one half inch needles into the animal at a point approximately mid-way between the upper and lower right quadrant. The short needle was used to minimize any possibility of accidently perforating the intestines. All injections were concluded without any undo incident. The two control animals were injected intraperitoneally with sterile isotonic saline solution at a volume of 1cc per 100 grams of animal in an identical manner. The sterile 5cc syringes and disposable needles were obtained from Central Supply, University Hospital, Ohio State University, School of Medicine.

Following the injections, the animals were observed at daily and nightly intervals for overt signs of toxicity for a period of fourteen days. These observations included investigation of the physical status of the animals before and after excitation. Excitation included physical probing and insufflation of the animals. Gross examination of the urine of each animal during these intervals was made for the presence of hematuria. Individual animal weights were recorded on the 3rd, 6th and 12th day of the experiment.

Animals were selected to be sacrificed on the basis of marked physical debilitation and unresponsiveness at any time over the period of the fourteen day experiment. The animals selected for sacrifice were on the following experimental days: 7 hours, 24 hours, 3days, 4 days, 6 days, 9 days, 10 days, 13 and 14 days. The period of 8-13 days represented the time following the initial injection when natural animal deaths occurred.

The animals were poleaxed and the abdomen was opened by a midline cross incision. The organs, including the lungs were examined to determine any gross organ changes. The bladder was carefully examined *in situ*, the ureters were cut and the bladder removed. Following bladder removal a dissection of the bladder was made exposing the mucosal bladder surface. A small portion of the mucosa was cut out for osmium tetroxide fixation. The remaining bladder was sectioned then cut into two portions and placed in buffered formal calcium. These tissues were then processed through fixation and dehydration in a Technicon, embedded in parrafin and sectioned to a thickness of 7-10 microns. The sectioned tissue was placed on individual glass microscope slides and stained with hematoxylin and eosin.

The small section of bladder (3-5 mm^3) cut for electron microscopy was finely sectioned with acetone cleaned razor blades to a thickness of approximately 0.5 to 1 cubic millimeter and placed *immediately* into a one percent isotonically prepared solution of osmium tetroxide containing glucose buffered in phosphate to a pH of 7.3 (Millonig, G., 1961). At exactly one hour following fixation in osmium tetroxide the osmium was removed with a stoppered pipette and the tissue dehydrated through 50%, 70%, 95% and absolute alcohol. Following this dehydration process, propylene oxide was added to the tissue which was then placed in the refrigerator for thirty minutes. Following this refrigeration the tissue was placed in equal volumes of Maraglass epoxy resin and propylene oxide for one hour and a half in the refrigerator. The addition of propylene oxide allows for adequate miscibility of the

epoxy resin and tissue which would otherwise not occur in alcohol. At the completion of this time interval the tissue was placed in Maraglass epoxy resin overnight in the refrigerator to allow for complete infiltration of the tissue by this embedding material. On the following day twenty-two individual tissue sections of each animal were placed in separate plastic polymerization capsules containing the Maraglass epoxy resin, trapped air bubbles were removed and the capsules placed in a 57-60°C oven for four days in order to achieve resin polymerization. Following polymerization, the plastic capsules were cut away and the tissue blocks sectioned with a glass knife on a Porter-Blum ultrathin microtome to a thickness of one micron. The sections were placed on glass microscopic slides and stained for approximately 30-45 seconds in a two percent solution of Toluidine Blue (Trump, B., et al., 1961) and then examined microscopically for the presence of bladder epithelium. Those blocks containing epithelium were set aside and the appropriate blocks for subsequent electron microscopy were selected on the basis of epithelial necrosis, dysplasia and evidence of cellular regeneration. Those blocks selected for electron microscopy were sectioned to a thickness of 200 Å with a glass knife on the Porter-Blum ultrathin microtome and expanded to maximum size by chloroform vapor applied over the sections. These expanded sections were placed on 300 mesh copper grids and allowed to dry on the grids for approximately twenty-four hours. The grids were then stained with a saturated solution of 50% alcohol and uranyl acetate for one hour

and a half in the dark (Watson, M., 1958), followed by application of lead citrate (Reynolds, F., 1963) for thirty minutes. The sections were examined on a RCA-EMU-3F electron microscope in the Department of Anatomy, The Ohio State University, School of Medicine.

RESULTS

I. Observations during the Life of the Animals

Following the intraperitoneal injections of cyclophosphamide, the animals were observed hourly for any toxic manifestations for the first eighteen hours. One hour following injections all the animals were responsive, but there was evidence of "ruffling" of their hair coat. Three hours following injection one animal had developed a marked hematuria and was moderately unresponsive. At six hours, twenty-five of the thirty experimental animals had developed marked, grossly observable hematuria. At seven hours the animal that had developed gross hematuria at three hours demonstrated a marked unresponsiveness to physical stimuli and was subsequently sacrificed. Two additional animals also demonstrating marked unresponsiveness were sacrificed at twenty-four hours. At the 30 hour period all animals (normals excepted) showed a moderate to marked degree of unresponsiveness following physical probing and insufflation. At 48 hours there was a moderate degree of improvement in terms of animal responsiveness in all but three animals. Sixty hours following injection all grossly observable evidence of hematuria had disappeared from the remaining animals and the urine was clear yellow. Three days following injection there was no change in the condition of the animals; the three animals previously described at 48 hours as being relatively unresponsive had remained so, with two being sacrificed on the third day and the other sacrificed on the following day. Cel-

culation of the weights of these animals at 72 hours showed weight loss in all animals ranging from 3 to 17 per cent (Table I). Five days following injection hematuria was evident for the first time in one animal and had recurred in another. On the sixth day following injection thirteen of the remaining twenty-four animals showed hematuria. All but one of these animals had previously demonstrated hematuria at six hours following injection. The animals themselves, however, appeared moderately responsive and calculation of their individual weights at this six day period showed an additional decrease ranging from 5-16 per cent in twenty-one of the animals and an increase of approximately three to five per cent in weight, as compared to their weights calculated at 72 hours post injection period, in the remaining three animals. The hematuria described in these animals continued intermittently over the next five days but had ceased in all but two animals by the twelfth day. Generally, all animals had shown a moderate degree of responsiveness from the sixth day post injection until the eighth day. On the eighth day the first animal was found dead and the responsiveness of some of the other animals had deteriorated. One animal found to be markedly unresponsive to physical stimuli at this time was sacrificed. Deterioration in some animals continued over the next five days resulting in additional natural deaths of three animals on the ninth day, two animals on the tenth day and one on the thirteenth day. At these time intervals of nine, ten and thirteen days, one animal was selected to be sacrificed. All of these animals selected were markedly unresponsive and appar-

ently near death. Calculation of individual animal weights at the twelfth day following injection indicated that there was weight loss ranging from 1-16 per cent in eleven animals, weight gain in one animal and identical weights in two animals, when compared to their calculated weights at the six days post injection period. Observance of the other animals over this period of time showed a general increase in responsiveness and activity. On the fourteenth day no remaining animal demonstrated any evidence of hematuria and all appeared increasingly responsive to physical stimuli. In order to study electron microscopically any evidence of bladder regeneration, one animal was selected at this fourteen day period to be sacrificed.

In conclusion, observations of thirty male Westar rats following a single intraperitoneal injection of 222 mg/kg of cyclophosphamide indicates the following:

1. Toxic manifestations as early as three hours following injection in the form of hematuria,

2. Widespread hematuria affecting 25 of the 30 animals within six hours along with marked decrease in responsiveness in all animals 36 hours following injection,

3. Cessation of gross hematuria after 60 hours followed by recurrance at periodic intervals in some animals,

4. Natural deaths occurring 8 to 13 days following injections with the maximum number of deaths occurring on the 9th day,

5. Evidence of animal rehabilitation in some animals on the twelfth day and in all the remaining animals by the 14th day as

evidenced by the lack of hematuria and increase in responsiveness to physical stimuli.

That these effects were the result of cyclophosphamide is evidenced by comparison with the control group of animals in which progressive weight gain occurred with no hematuria or loss of responsiveness following physical stimulation over the entire experimental period.

II. Microscopic Investigation

A. The Normal Bladder of Rats

The bladder is a hollow muscular organ which varies in shape and in size depending on the amount of urine it contains (Woodburne, A., 1961). In the rat there are five pairs of organs, surrounding the bladder, which are much larger than the corresponding organs in man. The organs consist of one pair of prostate glands, two large hook-shaped and convoluted seminal vesicles and along the concavity of these vesicles are the coagulating glands (Farris, E., et al., 1949). The mucus membrane lining the bladder is loosely attached to its musculature over most of its surface area and this appears wrinkled or folded except when the bladder is distended. The arteries of the bladder are the superior and inferior vesicles originating from the anterior trunk of the internal iliac artery. A dense network of veins surrounds the neck of the bladder in the endopelvic areolar tissue (Woodburne, A., 1961). The wall of the urinary bladder is composed of the same elements as that of the lower part of the ureter. This composition consists of transitional epithelium which varies in thickness according to the degree of distension, the tunica propria, smooth

muscle and adventitia (Hoskins, M., et al., 1952).

Light photomicrographs of formalin and osmium fixed normal bladder epithelium of the rat (Fig. 1) showed that the transitional epithelium consisted of several layers, with the surface cells larger and somewhat more flattened than the intermediate or basalar layers. Occasional dense bodies or so called "lipoid granules" were often present in the surface cells but rarely in the intermediate layers. The cellular configuration of the intermediate and basalar layers appeared somewhat polygonal. The basement membrane beneath the basalar cell layer was thin and not distinct. The connective tissue of the lamina propria was somewhat dense and in the deeper tissue areas, the smooth muscle appeared very thick.

Electron photomicrographs of the collapsed bladder epithelium of the normal rat were consistent with reported observations in the literature (Leeson, R. 1962; Richter, W., et al., 1963; Porter, K., et al., 1963). The cell types representing the transitional epithelium of the rat bladder consisted of a superficial cell which border the bladder lumen, an intermediate cell type which appeared slightly smaller than the superficial cell type, and a basal cell which rested upon a thin basement membrane (Fig. 2). The superficial cells were somewhat flattened and possessed a marked irregular or "scalloped" appearance at the lumenal surface, (Fig. 3). Within the superficial cell and in the proximal areas of the intermediate cell layers were numerous round and compressed vesicles (Figs. 3, 4 and 5). The cytoplasm of these transitional epithelial cells contained a few small,

relatively electron dense mitochondria, occasional dense bodies, and a fine fibrillar component seen to its best advantage in Figures 3 and 4. The plasma membrane was highly interdigitated with a terminal bar prominent at the luminal surface (Fig. 5). The Golgi apparatus was relatively inconspicious in most cells (Leeson, R., 1962) and the endoplasmic reticulum was present as rough-surfaced cisternal elements (Porter, K., et al., 1963) (Fig. 6). Small, relatively uniform ribosomal particles were present in the superficial cell (Figs. 4, 5, and 6) as well as the intermediate and basilar cell components. The intermediate cellular layer contained the compressed and round vesicles in areas just proximal to the surface epithelial cell and relatively the same quantity of cytoplasmic organelles (Figs. 2 and 5). Occasional small dense bodies were also seen in these cells. The basal layer did not have the compressed vesicles. The nuclei of these cells were smaller than the nuclei of the surface or intermediate cells and were considered to be the least characteristic of the three cell types (Leeson, R., 1962). The basal cells rested on the basement membrane which divided the epithelial cell layers from the lamina propria (Fig. 2). The lamina propria consisted of relatively dense connective tissue composed of collagen fibres surrounding fibroblastic cellular components and capillaries (Fig. 7).

B. Cytopathology of the Rat Bladder Epithelia after Cyclophosphamide

The first animal was sacrificed seven hours after the intraperitoneal injection of 222 mg/kg. This animal was the first of the experimental group to demonstrate evidence of gross hematuria which occurred three hours following injection. At the time of death, the

the animal had a marked lack of responsiveness to physical stimuli. The distended bladder *in situ* showed no evidence of hemorrhage. The lumen of the dissected bladder was filled with clotted and unclotted blood. Microscopically the bladder had a three plus edema and evidence of hemorrhage. The epithelium was intact in some areas but evidence of exfoliation was noted in other areas. Microscopic examination of the toluidine blue one micron sections indicated no remarkable cellular change (Fig. 8). The tissue was not observed in the electron microscope.

Twenty-four hours following injection two animals, which were not responsive to physical stimulation were sacrificed. Both of the animals had developed gross hematuria six hours following injection, with the hematuria persisting until death. *In situ* examination of the abdominal and thoracic cavities indicated no gross abnormalities although the kidneys in one animal were pale. There was no evidence of hydroureter. The bladder indicated no areas of hemorrhage, but the lumen contained fresh blood and blood clots. Microscopic examination of the hematoxylin-eosin and toluidine blue sections indicated two plus edema, evidence of hemorrhage and marked epithelial desquamation (Figs. 9 and 10). The kidneys had a slight amount of tubular damage.

Electron microscopic examination of the osmium tetroxide fixed bladder sections demonstrated some areas of hemorrhage as evidenced by the presence of red blood cells and fibrin along with inflammatory cells and products of cellular degeneration (Fig. 11). Numerous

extremely electron dense particles of various sizes surrounded by a single unit membrane, were observed within the neutrophils. These particles are interpreted as possibly being phagocytized granules associated with other ruptured and degenerating granulocytes (Fig. 12).

The epithelial cells contained a large number of granules that were irregular in shape and surrounded by a single membrane (Figs. 13 and 17). These structures are considered to be lipid granules. The cells lining the lumen of the bladder had only occasional compressed vesicles similar to those in the cells of the control group (Figs. 17, 19 and 20). There was an increased number of small, irregularly-shaped round vesicles in the cells lining the lumen and in cells beneath them (Fig. 13 and 17). These smaller vesicles could represent a morphological alteration of the compressed vesicles seen in the normal epithelial surface cells. The endoplasmic reticulum consisted of agranular cisternal and vesicular elements (Figs. 13 and 14). Occasionally small whorls of agranular endoplasmic reticulum were present (Fig. 15). In some cells, granular endoplasmic reticulum was also present, (Fig. 16). The Golgi apparatus in some cells was prominent, (Fig. 14) and portions of its components appeared swollen (Fig. 17). The mitochondria of some of these cells were normal although they were less electron dense than those described in the normal cells (Fig. 13). In other cells some of the mitochondria appeared damaged (Figs. 16 and 18). Some cells had irregularly shaped structures, which were surrounded by a single or double membrane and which were considered to be products of cellular degeneration

separated from the viable cell components (Figs. 17, 19 and 20). Dense bodies of various sizes (Figs. 14, 16, 19 and 20) were evident in some of the cell types. The plasma membrane was interdigitated throughout these cells with occasional evidence of terminal bars near the luminal surface (Fig. 13). In isolated areas the intercellular spaces were greatly increased (Figs. 15, 16, and 19).

Three days after injection of cyclophosphamide marked deterioration was evident in three animals. Two of these animals were sacrificed on the third day and the other animal was sacrificed on the fourth day after injection. In situ examination of the organs of the peritoneal and thoracic cavities indicated abnormalities only in the bladder. In all three animals in situ examination of the bladder showed large hematoma appearing masses on the surface and small focal areas of hemorrhage in the lumen. There was no grossly observable hematuria in these animals at the time of death. Microscopic examination of the hematoxylin-eosin and toluidine blue sections indicated a very similar pathology. The changes consisted of a two to three plus edema, submucosal hemorrhage including vascular necrosis and specifically, in the animal sacrificed four days after injection, damaged epithelium with the possible suggestion of regeneration (Figs. 21 and 22).

The two most conspicious characteristics of the bladder epithelial cells in these four day animals were marked degree of hemorrhage and a diminished cell cohesion which created large extracellular spaces between the cells (Figs. 23, 24 and 25). Also characteristic of these cells was a cytoplasmic disorganization probably due to the increased

exfoliative tendency of these epithelial cells. Numerous very small vesicles were present throughout the cytoplasm and the fine fibrillar components seen in the normal epithelium was inequally distributed within the cytoplasm of these cells (Fig. 23 and 24). Numerous erythrocytes were present in the intercellular spaces (Figs. 23 and 24). Small microvilli were seen along the surfaces of many cells with consequent release into the extracellular spaces (Fig. 24). Within the cytoplasm of some cells were multiple deposits of irregularly-shaped electron dense material (Fig. 25). This material did not seem to be membrane bound and its nature is unclear. Degenerating mitochondria were present in some cells (Figs. 26 and 27). Multinucleation occurred in some cells (Figs. 24, 30 and 31). The endoplasmic reticulum was increased in many of these cells in the form of cisternal granular elements (Figs. 28, 29, 31, 32 and 33). Occasionally 'myeloid-like" figures composed of smooth double membranes and surrounding cytoplasmic components were observed (Fig. 29). Some of the cells contained numerous round vesicles and dense bodies of varying sizes and shapes, (Figs. 30, 31 and 32), intensive Golgi complexes and long fusiform mitochondria (Figs. 31 and 32). It is of further interest to note that there was a total absence of the compressed vesicle component associated with the surface and intermediate normal cell types in all of these cells. Within the connective tissue component and interspersed between the collagen fibers were capillaries whose endothelial cells had a marked increase in organelle components. Some of the fibroblasts had altered mitochondria, endo-

plasmic reticulum and uniformly homogeneous aggregates of electron dense material surrounded by a single unit membrane (Fig. 34).

Eight days following injection the first of the experimental animals died. Evidence of deterioration in some of the animals was evident at this time by their lack of responsiveness to physical stimulation. One animal that was markedly unresponsive and hematuric was sacrificed on the eighth day following injection. The abdominal and thoracic cavities had no obvious gross abnormalities. *In situ* examination of the bladder indicated focal areas of hemorrhage along the bladder surface. The lumen of the bladder contained fresh and clotted blood. Microscopic examination of the bladder tissue sections indicated one plus edema, evidence of hemorrhage and multilayered, regenerating dysplastic epithelium (Fig. 35). The bone marrow was hypoplastic with evidence of debris and fibrin.

The electron photomicrographs of this bladder tissue showed necrotic and regenerating cells. In some cells (Figs. 36 and 37), the plasma membrane had ruptured and the cellular contents were expressed into the lumen. The nuclear membrane was broken in many areas and much of the chromatin had dispersed to the edges of the nucleus. Numerous small vesicles of varying sizes and shapes occupied the bulk of the cytoplasm along with moderately large vacuoles. Some of these vesicles surround other smaller electron dense spherical bodies which were themselves bounded by a single unit membrane. These were interpreted as lysosomes and/or cellular attempts at sequestration of damaged cytoplasmic components. Evidence of endoplasmic reticulum

and mitochondrial alteration could be seen in the cytoplasm of the surrounding cells. In the most severly affected cells the mitochondria were swollen and appeared irregular (Figs. 38, 39 and 40). In some of the mitochondria there was notable absence of the cristae or the cristae are bent back against the inner mitochondrial membrane (Fig. 39). It is interesting to note that these mitochondrial atypicalities were not uniform throughout the cells and that there were mitochondria within the same cells which did not appear as severly affected (Figs. 38 and 41). In general, the mitochondria appeared larger and less electron dense than the mitochondria in the normal cells. Numerous round vesicles, some containing electron dense material were present in many of these cells. The endoplasmic reticulum in these cells was granular with some of the cisternal elements slightly swollen in some cells (Figs. 38 and 39). The intercellular spaces between the cells increased (Figs. 39, 40 and 42). Desmosomes and cytoplasmic tonofibrils were still present (Figs. 38 and 40). Within some of these altered cells dense bodies of varying sizes and shapes were observed (Fig. 41). Within the dysplastic multilayered epithelium there were numerous small apparently immature cells, with irregularly shaped nuclei and peripheral aggregates of chromatin (Figs. 43 and 44). Many long strands of granular cisternal endoplasmic reticulum and prominent Golgi complexes characterized some cells (Figs. 42, 43 and 44). Numerous cytoplasmic microvilli extended from the cell surface and interdigitated with the microvilli of adjoining cells (Fig. 44).

Nine days following injection 3 of the animals died. Of the

remaining 19, 6 evidenced no worsening of their condition, 4 appeared increasingly responsive to physical stimulation, and 9 appeared very unresponsive to the stimuli. Two of the latter group were sacrificed. Examination of one of the sacrificed animals indicated bleeding from the left ear, but dissection of the left side of the head and left ear failed to show the cause of the hemorrhage. Examination of the abdominal cavity indicated a pale appearing liver which was removed, fixed in formalin and sectioned for light microscopy. The bladder showed a moderate amount of yellow urine. The bladder tissue had no evidence of edema or hemorrhage and a questionable normal epithelium. The bone marrow was atrophic with fatty deposits and a relative absence of cells. The liver was described as showing evidence of cloudy swelling with no fatty metamorphosis (Fig. 45). In the second animal the kidneys and liver were pale in appearance and there was a large hemorrhagic area on the surface of the left testicle. Microscopically the kidneys showed tubular damage with evidence of red blood cells within the tubules (Fig. 46). The bone marrow was considered badly damaged with considerable hypoplasia of the bone marrow elements. The testicles showed a marked degree of hemorrhagic necrosis (Fig. 47). The bladder in situ showed no evidence of hemorrhage. Direction of the bladder showed a small amount of yellow colored urine. Microscopic examination of the hematoxylin-eosin and toluidine blue sections of the bladder from these two animals was essentially the same; zero to one plus edema, no hemorrhage and questionably normal, multilayered epithelium with some evidence of

dysplasia in certain areas (Fig. 48).

Electron photomicrographs of this bladder tissue indicated three major areas of cellular changes; cytoplasmic and nuclear vacuolization, change suggestive of cellular sequestration and dysplasia with regeneration.

The most remarkable feature in some of the bladder epithelial cells of the animals sacrificed at this time were the cytoplasmic and nuclear vacuolization of the surface cells. In Figures 49, 50 and 53, the surface cells with their protrusions of microvilli into the lumen showed large cytoplasmic and/or nuclear vacuolization. In Figure 49, there were two moderately large vacuoles in this surface cell with possible binucleation. Within these nuclei there were peripheral condensations of nuclear chromatin and the suggestion of an increase electron density. In Figure 50, there appears to be vacuole formation in addition to the established cytoplasmic vacuoles within this apparently active cell. The mitochondria in this cell were numerous and as a general rule, consistently longer than those described in normal bladder epithelium. That some of these vacuoles represented an attempt on the part of the cell to sequester out damaged elements can be seen in Figure 51. In this cell there was a large focal area of degenerating substances which was surrounded by a single and sometimes double unit membrane. At the same time the nucleus was somewhat electron dense and uninterruptedly surrounded by its unit membrane. Multiple nucleoli were seen and there was an increase in the rough surfaced endoplasmic reticulum. In Figure 52, additional

examples of vacuolar inclusions were present. Numerous organelles were seen in this cell including occasional vesicles of the compressed type as described in the normal surface epithelial cell. Remarkable mitochondrial alteration were not generally manifest in these cells but most changes chiefly consisted in an increase in size, length and configuration with some evidence of degeneration. In one area, however, surrounded by plasma membrane interdigitations were a cluster of large swollen degenerating mitochondria (Fig. 54). The endoplasmic reticulum was markedly increased in the majority of the cells over the normal epithelium in the form of rough or granular cisternal elements (Figs. 55, 56). The connective tissue component of the lamina propria had fibroblastic degeneration and necrosis (Fig. 57).

The second group of cellular changes was an apparent disunity between the cells extending all the way to the basement membrane (Figs. 58 and 59). No such disorganization was manifest in the sections of normal cells. In addition, those obviously exfoliating cells appeared to be undergoing degeneration as manifest by the numerous small dense bodies (Fig. 58) and nuclear fragmentation (Fig. 59).

There could be noted in other cells hyperactivity with numerous mitochondria, round vesicles, prominent Golgi complexes, and endoplasmic reticulum (Figs. 55 and 56). Numerous dense granules of various sizes were present in some of the cells along with cisternal elements of the granular endoplasmic reticulum (Figs. 56, 60 and 61). Cellular dysplasia and evidence of cellular regeneration was seen (Figs. 62, 63 and 64). Nuclear dysplasia was characterized by in-

creased electron density of the nucleus along with peripheral nuclear chromatin aggregations and multiple nucleoli. In addition, long filamentous elements of endoplasmic reticulum, suggesting increased protein synthesis, was present in many cells (Figs. 63 and 64).

On the tenth day following injection an additional two animals described on the ninth day as unresponsive had died spontaneously. Up to this time, six animals had died naturally and nine animals had been sacrificed. Five of the nine animals which were described as markedly unresponsive on the ninth day were still unresponsive but alive on this tenth day. Because of the two natural deaths at this time period one of these animals was sacrificed. Examination of the abdominal cavity showed a pale liver and kidneys. These organs were excised, sectioned and stained for light microscopy. In situ examination of the bladder showed areas of hemorrhage on the bladder surface. Dissection of the bladder showed a small amount of yellow, clear urine. Microscopic description of the kidneys indicated focal areas of tubular damage (Fig. 65). The liver showed focal areas of necrosis with chronic passive congestion. The microscopic description of the hematoxylin-eosin and toluidine blue bladder sections showed a one plus edema, one plus hemorrhage and the suggestion of healing by the appearance of a multilayered epithelium. In addition, an amorphous appearing material was evident between the cells (Fig. 66).

Electron microscopically the cells of this multilayered epithelium were relatively unremarkable except for the presence of the intercellular spaces which has been described in all of the animals up to this time (Fig. 67). Noticeably present in these cells were numerous

desmosomes and cells showing multiple and prominent nucleoli. In some areas of this multilayered epithelium were cells in which the extracellular spaces were larger and had begun to fill (Fig. 68). The fine fibrillar material of some of the cells was seemingly aligned along the cellular margin adjacent to the plasma membrane interdigitations (Fig. 69). This "filling process" is seen to better advantages in Figure 70, which shows a marked increase in polyribosomal rosette formations along with occasional mitochondria which are irregularly shaped. Elements of rough endoplasmic reticulum and the fine fibrillar component were also in evidence. Figures 71, 72, and 73 show the extent of this extracellular filling with moderately electron dense material along with prominent desmosomes. In Figure 72, numerous round vesicles are in appearance along with prominent Golgi areas. The nuclei in all of these cells appeared very active with increased nuclear densities, peripheral chromatin aggregations and prominent nucleoli. Figures 74 and 75 show portions of cells in which some of the mitochondria were noticeable swollen and distorted, the endoplasmic slightly swollen and somewhat irregular, and numerous round vesicles and dense bodies of varying shapes and sizes. The attempt at cellular sequestration is evident in Figure 76 where a large spherical electron dense substance is being segregated in the cytoplasm of the cell, along with other degenerating cellular material, by a single unit membrane. With seemingly increased frequency are the numbers and predominance of the Golgi complexes in many of these cells as seen in Figures 77 and 78. It's significance is unclear,

but one may perhaps suggest that there might be a relation between the cell's increased secretory capability as manifest by such a predominence of the Golgi systems and the extracellular filling evident in many of these cells. I became interested in observing as to which direction this material is going, i.e. from the surface forwards to the basement membrane or from the submucosal areas forward to the surface cells. In Figures 79 and 80 numerous intercellular filling areas were noticed near the surface cell. The higher magnification of Figure 80 shows microvilli which seem to arise from a "bubbling" off the surface cell with consequent liberation of the former cytoplasmic component into the lumen. In addition, there is present a mucoid-like aggregation at the surface layer of these cells which was interpreted as lipid. Figure 81 shows a "fluffy" electron dense material within and partially obscuring the collagen fibers of the connective tissue component of the lamina propria. This material, in more dilute form might represent approximately the same electron denseness as seen in the intercellular spaces. Figure 82 shows at higher magnification an apparent communication between the plasma membrane and a somewhat dissolved or obscured basement membrane. Consequently the question as to the origin and flow of this material remained unsolved.

On the thirteenth day following injection fourteen animals remained alive. Eleven of the fourteen showed increased activity and three remained moderately unresponsive. During the thirteenth day one of these unresponsive animals died and another was selected to be

sacrificed. This animal had had intermittent episodes of hematuria through the twelfth day but there was no evidence of hematuria at the time it was sacrificed. The animal that had died naturally had episodes of hematuria through nine days after injection but had shown no hematuria at the time of death. Examination of the abdominal and thoracic cavities indicated no abnormalities and further examination of the bladder both *in situ* and in the excised state indicated it to be essentially normal. Light microscopic examination of the bladder tissue sections showed no evidence of edema or hemorrhage with dense layers of possible regenerating epithelium (Fig. 83).

Electron photomicrographs of this multilayered epithelium indicates areas of small polygonal, spherical cells approximating the size of the normal basal epithelial cell but containing more organelle components (Figs. 84 and 85). These nuclei appeared somewhat hyperactive with prominent single and multiple nucleoli. The mitochondria were relatively smaller than those described in the previous experimental groups but were seemingly increased in numbers over the normal cell component. Empty intercellular spaces were still very much in evidence along with multiple desmosomes, and occasional dense bodies (Figs. 85 and 86). The cells lining the basement membrane appeared to be longer as compared to the polygonal, spherical cells previously described (Fig. 87). At higher magnifications, elements of the granular endoplasmic reticulum and small mitochondria could be seen in this elongated cell type as it rests on the basement membrane adjacent to the connective tissue component of the lamina

propria (Fig. 88). Relatively less frequent but still occasionally present were local areas of cellular degeneration as evidenced by small extremely electron dense deposits of material surrounded by a single unit membrane. In addition, whorls of smooth membranes and somewhat swollen mitochondria were occasionally present (Fig. 89). In some occasional cells there was evidence of cell shrinkage with condensation of the altered organelle components in the form of swollen mitochondria, prominent Golgi zones and somewhat enlarged cisternal elements of the granular endoplasmic reticulum (Fig. 90).

Fourteen days post injection all of the remaining twelve animals were responsive to physical stimulation including the one remaining animal described at thirteen days as moderately unresponsive. This animal was selected at this fourteen day period to be sacrificed. Examination of the abdominal and thoracic cavities indicated no observable changes in the organs including the bladder. Light microscopy of the bladder tissue indicated a one plus edema and hemorrhage with a multilayered somewhat dysplastic epithelium (Fig. 91).

Electron microscopy of this bladder epithelium indicates numerous small cells with prominent, electron dense nuclei (Fig. 92). Occasional dense bodies were seen along with normal appearing electron dense mitochondria. Occasional binucleation was noted in some of the surface cells (Fig. 93) which also contained numerous compressed ar round vesicles. Occasional evidence of cellular degeneration could still be observed among the normal cells (Fig. 94). There were still areas along the plasma membrane in some cells which showed increased intercellular spaces (Figs. 95 and 96). However, in the vast

majority of cells, Figures 96 and 97, the plasma membrane interdigitated closely with the absence of large intercellular spaces. The most conspicious characteristic of many of these moderately dysplastic cells is the remarkable increase in the rough endoplasmic reticulum and mitochondrial organelle components (Figs. 97 and 98). In Figure 98 and at a higher magnification (Fig. 99), long strands of endoplasmic reticulum appeared in close proximity to the mitochondria culminating in some cells in a whorl-like arrangement. Occasional small vacuoles were also seen in some cells along with dense bodies, ribosomes, and fine fibrillar components (Figs. 97, 98, and 99). The fibroblastic components of the connective tissue appeared normal with only the suggestion of a small amount of edema (Fig. 100).

DISCUSSION

Cyclophosphamide when introduced into animals or man is converted from an "inactive" to an "active" cytoxic derivative(s) (Des Pres, J.D., 1960). The mechanism of this transformation is considered to be through enzymatic action of phosphamidases or acid phosphatases (Coggens, P.R., et al., 1960). The rat bladder epithelium has high phosphamidase activity (Meyer and [illegible], et al., 1957). Animals receiving intraperitoneal injections of cyclophosphamide develops an acute cystitis similar to the human side effects reported in the literature during and following cyclophosphamide chemotherapy. However, direct injections of the inert cyclophosphamide into the bladders of experimental animals (Phillips, et al., 1961) did not cause a cytotoxic effect and suggests the formation of the cytotoxic derivatives probably at multiple sites in the body.

Electron micrographs of transitional bladder epithelium of Wistar rats following a single intraperitoneal injection of 222 mg/kg of cyclophosphamide illustrate four general cytologic changes which were evident to varying degrees over a fourteen day experimental period. The first of these cytologic alterations, cellular degeneration, was most noticeable within the cytoplasm of the bladder epithelium of the animal sacrificed twenty-four hours after injection. The second cytologic characteristic, cellular exfoliation and cell death, occurred predominantly in the animals sacrificed four, eight and nine days after injection. Cellular dysplasia, most notable affecting the

nucleus, and cell regeneration were the other cytologic representations observed. Cellular dysplasia and limited cellular regeneration occurred as early as eight days after injection. Regenerating normal epithelium was the dominant characteristic in the animal sacrificed fourteen days after injection.

In the animals sacrificed twenty-four hours after injection, irregularly-shaped electron dense granules surrounded by a single unit membrane was observed within the granulocytes located in the areas of marked cellular degeneration. It is felt that they represent phagocytized granules liberated from other cells of the granulocytic series which were undergoing degeneration. Hodson, P.R., 1961, described an increased granulocytosis in interstitial cystitis and ulcerative colitis following the administration of diphtheria toxoid. These granulocytes reached a maximum concentration after twenty-six hours. These cells had polymorphous nuclei, cytoplasmic edema and "shedding of granules with mononuclear ingestion". Itoga, et al., 1962, described the presence of cytoplasmic inclusion bodies, perhaps identical to the Döhle bodies described in some pathological states, along with other "coarse dark staining granules" occurring singly, or in the form of multiple inclusions within the granulocytes of patients undergoing treatment with cyclophosphamide. These inclusion bodies were located in the periphery of the cytoplasm and occasionally protruded beyond the normal cell contour. The number of the Döhle bodies, the biochemical basis of which is unknown, increased with decreasing maturation of the granulocytes during cyclophosphamide chemotherapy.

This suggested to Itoga that "cyclophosphamide interfered with nuclear and cytoplasmic maturation reflecting a basic disturbance of nucleic acid metabolism".

The most distinctive characteristic of the normal transitional epithelium was the presence of compressed and round vesicles in the superficial and intermediate cell layers. Compressed vesicles have been observed only within transitional epithelium (Rhodin, J., 1963). However, their morphologic appearance seems to vary in certain animal species. Electron micrographs of the superficial cells of bovine transitional bladder epithelium (Kamczak, N., 1964) illustrated a spherical appearance of these cytoplasmic vesicles as opposed to the long, fusiform appearing vesicles present in rat and mouse bladder epithelium (Walker, B.F., 1960; Leeson, R., 1962). Batifor , H., et al., 1964, described in human bladder transitional epithelium numerous smooth-surfaced intracytoplasmic vesicles, some of which were compressed into "slits".

Several functions have been suggested for these vesicles. Rhodin, 1963, stated that their function is unknown but suggested that they might be dissolved crystals which migrate to the luminal surface. Walker, B.F., 1960, postulated that these compressed vesicles might serve as a dynamic urine barrier by carrying fluids from the cytoplasm to the cell surface. He stated further that there was a similarity of the compressed vesicles of transitional epithelium with those seen in the protozoan, Tetrahymena infusiorum, which was purported to be responsible for movement of cytoplasmic fluid to the contractile vacuole

(Rudzinska, M.A., 1958). Porter, K., et al., 1963, suggested that the compressed vesicles might be formed at the surface of the superficial cell where adjacent cytoplasmic [illegible] and the cytoplasm between them became pinched off into the cytoplasm. They showed that the limiting membranes of these vesicles are 100 Å in diameter and possess a trilaminar membrane structure identical to that of the plasma membrane. They further suggested that these compressed vesicles might serve as plasma membrane storage areas necessary for cellular bladder expansion. Walker, B.F., 1960, demonstrated that injection of thorium dioxide into the bladder lumen of mice terminated in the presence of thorium crystals within the round vesicles of the surface cells and later in the connective tissue component of the bladder. At no time were these crystals observed in the compressed vesicles. Therefore the origin of the compressed vesicle would not seem to be formed from a "pinching off" or "compartmentalization" at the luminal surface which would result in vesicle formation, but would suggest the existence of the vesicles within the superficial and intermediate cells themselves. Lesson, R.C., 1962, observed electron microscopically that there was an increase in the number of compressed and round vesicles within the superficial cells of the rat transitional epithelium following the administration of distilled water into the bladders of these experimental animals. However, no changes occurred following the administration of hypertonic and isotonic solutions into the bladder of other experimental animals. These results were explained on the basis that in such a hypotonic environment, fluid would enter

the cell leading to the appearance of large numbers of vesicles, and would support the theory of Walker, B.F., 1960, that the nature of these vesicles was excretory in function, i.e., that they originate as vacuoles within the cytoplasm and pass to the luminal surface. In Figure 3 of the normal rat superficial bladder epithelial cell, some of these compressed vesicles appear to have ruptured at the surface of the superficial cell. This would support the theory of Walker that the compressed vesicle moves to the cell surface.

One of the most significant observations of the transitional epithelium in the treated experimental animals was the marked reduction in the number of the compressed vesicles and an increase in the number of various sized round vesicles. These alterations occurred in the treated animals with the exception of the animal sacrificed at fourteen days. In this animal the normal epithelial constituents were again present. In the twenty-four hour animal small and irregularly-shaped round vesicles were observed along the surface of the cell bordering the lumen and might represent increased pinocytotic activity of the surface cells. Coincidently, or significantly related to the reduction in numbers of the compressed vesicle, was the appearance of marked enlargement of intercellular spaces, in the animals sacrificed over the specific time periods, except after fourteen days. It had been mentioned earlier that the dimensions of the apparent trilaminar lined vesicles are identical to that of the plasma membrane (Porter, K., et al., 1963). If the function of these vesicles could be cellular excretion and also plasma membrane storage, the disappearance of these

vesicles would then deplete the amount of storage plasma membrane. This failure in the replacement of plasma membrane might result in weakened conditions along the existing plasma membrane which might terminate in cellular compression with the resultant increased intercellular spaces.

Alterations observed in the organelle component of some of these cells twenty-four hours following the injection of cyclophosphamide were the increase in the numbers of dense granules, the presence of pooled lipid aggregations within the cytoplasm, the disassociation of the ribosomes from the endoplasmic reticulum, and damaged mitochondria. In a study of the histological effects of nitrogen-mustard on tumor tissue, it was observed by light microscopy that there was a reduction in the numbers of cells in mitosis, a "ballooning of the cytoplasm" by fat deposits and the presence of nuclear fragmentation (Spitz, S., 1948).

A review of the literature fails to show previous work at the ultrastructual level concerning the cytopathologic changes in damaged transitional epithelium. However, a great deal of work at this level has been accomplished in other tissues, notably the liver, following the administration of cytopathologic agents. The cytopathologic changes in the liver reported by others (Farber, E., 1963, 1964; Reynolds, E.S., 1963) were the result of the administration into laboratory animals of ethionine and carbon tetrachloride respectively. Some of the resultant ultrastructural changes occurring in these livers were similar to those changes observed in the transitional epithelial

cells of the experimental animals following cyclophosphamide injection. These changes consisted of the disassociation of the ribosomes from the endoplasmic reticulum, the presence of lipid aggregations in the cytoplasm, and evidence of altered and damaged mitochondria. Direct correlation in the ultrastructural changes between two such divergent and functionally different organs and different cytotoxic agents cannot be established. However, the suggested explanations for the cellular changes due to treatment with the various cytotoxic agents may be applicable to understanding the changes due to cyclophosphamide. The applicability of such changes to the bladder, however, depends on future cytochemical and cytophysiological studies which are beyond the scope of this thesis.

Farber, E., et al., 1963, 1964 studied the effect of ethionine, the ethyl analog of the naturally occurring amino acid methionine, in the liver, and further noted that similar reproducible lesions could also be demonstrated in the pancreas, kidney and testis. Some of the major alterations of the liver cells following ethionine administration were the inhibition of protein synthesis demonstrated both in vivo and in vitro, and the occurrence of fat deposits within the cytoplasm of the liver cell. Related to this decrease in protein synthesis and increased triglyceride concentration within the cells was the decrease in total adenosine triphosphate (ATP). The presence of triglyceride levels within the cells of the liver was explained on the basis of the formation of S-adenosylethionine by the action of ethionine and ATP. This compound which is poorly metabolized results in a "trapping of adenine" which overtaxes the ability of the cell to manufacture

this purine component. The reduction in adenine would effect the synthesis of ATP which would normally furnish the necessary energy for the formation of the protein moieties and in addition, possibly limit the export of the triglycerides and lipoproteins out of the cell and into the blood plasma. The administration of adenine or ATP to this system prevented the accumulation of excess triglycerides within the liver cells.

It is of interest to note in this connection that when formate, labeled with C^{14} was incorporated into the purines of RNA and DNA in sensitive and resistant cyclophosphamide tumors, there was an inhibition of DNA adenine by cyclophosphamide in the sensitive tumor. In addition, the major cytologic effect of susceptible cells following cyclophosphamide administration was the reduction in protein synthesis (Strosier, V.J., 1962, Wheeler, G.P., 1962, Maguire, H.C., 1961, and Kovacs, S., 1960).

Reynolds, E.S., 1963 studied the effect of the non-ploar lipid solvent, carbon tetrachloride, on rat liver. His obeervations of cellular changes included vacuolization of the cisternae of the endoplasmic reticulum, degranulation of its membranes and an increased number of free ribosomes. In addition, there were morphologic alterations of the mitochondria and swelling of the Golgi vesicles. As a non-polar lipid soluble substance, carbon tetrachloride was thought to transform the constituent bimolecular phospholipid leaflets of the cell membranes from a "crystalline" to liquid phase. Such shifts in the membranes physical-chemical properties might transform the sheet-

like membranes with low surface tensions to droplets and vesicles with high surface tension." Therefore, the alterations in cytoplasmic organelles may be manifestations of the "physical presence of the lipid solvent in the membranes of these organelles."

Thus, the lipid deposits present in the cytoplasm of rat bladder transitional epithelium following cyclophosphamide administration might result from: (1) the inability of the cell to metabolize nutrient fat material that might be transported into the cell. With some inhibition of the purine adenine perhaps there would be a drop in cellular ATP and therefore a reduction in the energy levels necessary for normal and adequate cell metabolism; (2) markedly altered cellular metabolism which culminates in the formation of neutral lipid; (3) degenerative processes of the cell due to the cytotoxic agents which might result in accumulation of lipid, perhaps from alteration of membrane phospholipid components. Kanczak, N., 1964, histochemically demonstrated areas associated with the lysosomal membrane component of the transitional epithelial cell to be phospholipid in structure.

The evidence of cellular degeneration in many of the cells of the experimental animal groups was accompanied by an increase in dense granules. The large dense granules in the normal bladder epithelial cells of the rat are lysosomes (Kanczak, N., 1964). These organelles which contain the hydrolytic enzymes acid phosphatase and -glucoronidase, were increased in many of the cells of the animals sacrificed at 24 hours, four, eight, and nine days. This is consistent with the ob-

servations of Van Lancker, J.L., et al., 1959, who noted the release of these enzymes from "cytoplasmic granules" in cells during the course of cell autolysis.

Spitz, S., 1948, in his studies of the effect of nitrogen mustard on tumor cells, stated that in all of the cases studied not all the cells were equally affected by the cytotoxic agent. Injections of cyclophosphamide into leukemic mice resulted in a delay of the pre-mitotic process with subsequent abnormal and slowly progressing mitosis in some cells (Kovacs, et al. 1960). At 48 hours following injection, the mitotic figures had the same chromosome complement as the tumor cells before therapy, indicating that the chromosome number of these cells was not affected. Electron microscopy of the bladder transitional epithelium of the rat is in agreement with these observations. Morphologic alterations of the cells following cyclophosphamide indicated agranulation of endoplasmic reticulum, swelling of the mitochondria, nuclear degeneration and karyolysis in some cells. However, in other cells, the granular endoplasmic reticulum was in long filaments, the mitochondria were increased in numbers and the nuclei, at the later time periods, appeared hyperactive and somewhat dysplastic.

Four days following injection the greatest amount of cellular morphologic changes were noticed. These changes were a marked degree of hemorrhage and diminished cell cohesion culminating in very large intercellular spaces. Evident at this time period were nuclear alterations in the form of irregularities in size and shape, increased elements of rough endoplasmic reticulum and "whorls" of agranular

membranes surrounding electron dense cytoplasmic components. The increased evidence of nuclear atrophy and cytoplasmic degeneration at this time in some cells is consistent with the observed cytologic effects of cytotoxic mustards observed at the light microscopic level (Klopman, H.R., et al., 1960).

The presence of the agranular whorls of membranes at this and at later times has been described as a mechanism of cellular cytoplasmic sequestration (Hruban, Z., et al., 1963). Focal cytoplasmic degradation, a cytoplasmic sequestration of damaged cytoplasmic components, represents a cytopathologic alteration which can be distinguished from total cytoplasmic degeneration and cell necrosis. In the early phases of focal cytoplasmic degradation portions of the cytoplasm are usually limited by a single membrane or several layers of "myeloid smooth membranes". These inclusions may contain altered cytoplasmic components such as pleomorphoric electron dense materials, granules of various sizes, lipid droplets, relatively intact mitochondria, ergastoplasmic or Golgi components (Hruban, Z., et al., 1963). Such examples of focal cytoplasmic degradation were observed in many of the cell types of the animals sacrificed four to thirteen days after injection. Conspicious in some of these cells were the concentric "myeloid membrane formations" or whorls of agranular membranes along with sequestered cytoplasmic elements surrounded by a single membrane. The myeloid configurations were similar to the formations described by Hruban, Z., et al., 1963, in cells which have "abnormalities in protein or cholesterol synthesis".

Evidence of cellular degeneration with alteration of the nuclei and cytoplasmic components occurred through the thirteenth day after injection. Cytoplasmic and nuclear vacuolisation was most evident at the ninth day and is consistent with the cytologic observations of Bierman, H.R., et al., 1960, of tumor cells from various effusions following cyclophosphamide chemotherapy. His observations in order of decreasing significance included increased numbers of degenerating forms, increase in nuclear vacuolisation, loss of cell membrane continuity and increased cytoplasmic vacuolisation.

The normal transitional epithelium of the rat possesses occasional intercellular spaces between adjacent basal cells (Kamenak, H., 1964). The significance of intercellular spaces as a cellular transport mechanism was reported in a study of the uptake of thorium dioxide within the intercellular spaces of corneal endothelium in the rabbit which terminated in the presence of the crystals within the corneal stroma, (Kaye, G., et al, 1962). Battifera, H., et al., 1964, considered the intercellular spaces in the human bladder transitional epithelium as a possible mechanism for the transport of water and solutes through the epithelium. Johnson, J.A., et al., 1951, using an isotonic solution of sodium chloride in heavy water, determined that there was a considerable exchange of water molecules across the mucosal bladder wall in dogs.

In the experimental animals following cyclophosphamide injection there were numerous intercellular spaces between adjecent cells in all layers of the transitional epithelium. With the exception of one

animal sacrificed ten days following injection, these intercellular spaces with the experimental procedures used for tissuespreparation appeared empty. In this one animal, however, there was focal areas of the epithelium in which these intercellular spaces were partly or completely filled with an amorphous, electron dense material. Histochemical stains such as the periodic-acid Schiff for mucopolysaccharides, Oil Red O for neutral fats, and basic fuchsin for protein deposits were negative. Thus, although the nature of this material was not determined, these deposits might represent further evidence of degeneration, altered cell metabolism and / or alteration in plasma membrane permeability which terminated in the "pooling" of material between the cells.

Nuclear dysplasia as described by the light microscopy and evidenced in the electron micrographs by increased nuclear densities and peripheral chromatin aggregations was evidenced in some cells as early as eight days and lasted to a limited extent until the thirteenth day. All of these cells had an increase in cisternal elements of rough-endoplasmic reticulum which at the height of regeneration on the fourteenth day became long, filamentous and, in many instances, existed in close apposition to increased numbers of small, dense and normal appearing mitochondria.

CONCLUSION

Injections of 222 mg/kg of cyclophosphamide into the intraperitoneal space of thirty white male Wistar rats illicited a severe hemorrhagic cystitis due to the pooling of toxic urine in the bladder. The observable toxic manifestations of active cyclophosphamide occurred as early as three hours post injection as determined by the presence of gross hematuria in one animal and subsequent weight loss in most of the remaining animals over the experimental period of fourteen days. Cytologic alterations examined electron microscopically consisted of the following:

(1) Cellular degeneration in the form of altered cytoplasmic organelles such as the endoplasmic reticulum and mitochondria along with increased lipid aggregations primarily in the animals sacrificed after twenty-four hours.

(2) Cellular exfoliation and cell death in the animals sacrificed at four, eight, and nine days.

(3) Cellular dysplasia most noticeably affecting the nucleus in the animals sacrificed from eight to thirteen days following injection.

(4) Marked evidence of epithelial regeneration in the animal sacrificed at fourteen days.

SUMMARY

Cyclophosphamide is a nitrogen mustard which is linked by way of the nitrogen atom to a phosphoric-acid-esteramidase that allows for its relative inactivity *in vitro*. Through enzymatic activity *in vivo* there is a transformation of the inert cyclophosphamide to active cytotoxic derivative(s) which causes cytologic alteration and cell death to normal and tumor tissues. Its value as an antitumor agent has been in the treatment of patients with malignancies arising from hematopoietic tissues, malignant lymphomas, leukemias and carcinomas arising from the breast and ovary. One of the side effects reported following cyclophosphamide chemotherapy was the occasional manifestation of a sterile, hemorrhagic cystitis.

Injections of cyclophosphamide into white male Wistar rats illicits a severe hemorrhagic cystitis. Electron microscopic examination of rat bladder transitional epithelial over various periods of time following injection, illustrated cytoplasmic degeneration, cellular exfoliation and cell death, nuclear dysplasia and subsequent epithelial regeneration.

TABLE

TABLE I

Animal Number	Weights Prior to Injection	Weights 3 days	Weights 6 days	Weights 12 days	Total Weight Loss/Gain %
1.	253 gr.	243gr.(↓ 7%)	Sacrificed at 3 days	-	↓ 7%
2.	260 gr.	244gr.(↓ 6%)	Sacrificed at 3 days	-	↓ 6%
3.	272 gr.	264gr.(↓ 3%)	251gr.(↓ 5%)	234gr.(↓ 6%)	↓ 14%
4.	277 gr.	Sacrificed at 7 hours	-	-	-
5.	267 gr.	263gr.(↓ 5%)	271gr.(↑ 3%)	266gr.(↓ 2%)	↓ 4%
6.	258 gr.	247gr.(↓ 8%)	237gr.(↓ 7%)	220gr.(↓ 4%)	↓ 19%
7.	294 gr.	246gr.(↓13%)	226gr.(↓ 8%)	202gr.(↓11%)	↓ 32%
8.	285 gr.	240gr.(↓16%)	203gr.(↓15%)	Sacrificed at 9 days	↓ 30%
9.	296 gr.	253gr.(↓11%)	263gr.(↑ 5%)	235gr.(↓12%)	↓ 19%
10.	299 gr.	269gr.(↓ 9%)	244gr.(↓ 8%)	234gr.(↓ 7%)	↓ 18%
11.	231 gr.	269gr.(↓ 6%)	240gr.(↓ 7%)	246gr.(↓ 1%)	↓ 13%
12.	281 gr.	265gr.(↓ 7 .)	223gr.(↓15%)	Sacrificed at 2 days	-
13.	259 gr.	Sacrificed at 24 hours	-	-	-
14.	306 gr.	246gr.(↓ 7%)	234gr.(↓ 6%)	Sacrificed at 10 days	↓ 13%
15.	264 gr.	231gr.(↓13%)	204gr.(↓11%)	Death at 10 days	↓ 23%
16.	283 gr.	256gr.(↓10%)	220gr.(↓11%)	Death at 10 days	↓ 21%
17.	274 gr.	241gr.(↓12%)	202gr.(↓16%)	Death at 7 days	↓ 27%
18.	279 gr.	246gr.(↓11 .)	227gr.(↓ 8%)	195gr.(↓15%)	↓ 30%

TABLE I Continued

Animal Number	Weights Prior to Injection	Weights 3 days	Weights 6 days	Weights 12 days	Total Weight Loss/Gain %
19.	273 gr.	247gr. (↓10%)	228gr. (↓ 8%)	Death at 8 days	↓ 17%
20.	270 gr.	236gr (↓12%)	216gr. (↓11%)	177gr. (↓16%)	↓ 35%
21.	342 gr.	302gr (↓12%)	290gr. (↓ 4%)	260gr. (↓10%)	↓ 24%
22.	259 gr.	235gr. (↓10%)	206gr. (↓13%)	206 (→)	↓ 21%
23.	284 gr.	263gr. (↓ 8%)	230gr. (↓13%)	229 (→)	↓ 20%
24.	263 gr.	244gr. (↓ 7%)	250gr. (↑ 3%)	274gr. (↑ 9%)	↑ 4%
25.	262 gr.	Sacrificed at 24 hours	-	-	-
26.	294 gr.	261gr. (↓11%)	228gr. (↓12%)	Sacrificed at 9 days	↓ 22%
27.	269 gr.	225gr. (↓17%)	198gr. (↓12%)	181gr. (↓ 9%)	↓ 33%
28.	264 gr.	226gr. (↓15%)	209gr. (↓ 8%)	Death at 8 days	↓ 21%
29.	282 gr.	244gr. (↓14%)	211gr. (↓14%)	Death at 9 days	↓ 26%
30.	272 gr.	245gr. (↓10%)	Sacrificed at 4 days	-	↓10%
NORMALS					
1.	277 gr.	303gr. (↑10%)	320gr. (↑ 6%)	345gr. (↑ 8%)	↑25%
2.	342 gr.	348gr. (↑ 2%)	350gr. (↑ 1%)	352gr. (↑ 1%)	↑ 3%

ELECTRO

PHOTOMICROGRAPHS

Figure 1. Normal bladder mucosa of the rat showing the large flattened surface epithelial cells and the smaller polygonal shaped intermediate and basal cell layers. X640.

Figure 2. The normal superficial, intermediate and basal cell layers of the rat transitional bladder epithelium are shown. Note the marked interdigitations of the plasma membrane and the occasional evidence of intercellular spaces in the areas adjacent to the basal cell. Note also the numerous compressed vesicles in the superficial and intermediate cell layers along with the occasional large dense granule. X 4,000.

Figure 3. Superficial cell of the normal bladder epithelium. Note the scalloped appearance of the cytoplasmic edges and the apparent remnants of ruptured compressed vesicles at the surface. X 35,900.

Figure 4. Intermediate cell area of the normal rat epithelium showing numerous compressed vesicles and fine fibrin structures within the cytoplasm. Note the small ribosomal aggregates, occasional round vesicles, and the small electron dense mitochondria. X 10,700.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Figure 5. Intermediate and superficial cell components of the bladder epithelium showing the compressed and round vesicles, occasional dense granules and occasional desmosomes along the interdigitating plasma membrane. Note the large terminal bar at the luminal surface adjoining two superficial epithelial cells. X 5,900.

Figure 6. Ribosome associated endoplasmic reticulum and electron dense mitochondria within the normal transitional epithelial cell of the rat bladder. Note the ribosomal aggregations, the fine fibrillar component and the prominent desmosomes with adjacent tonofibrils. X 29,600.

Figure 7. The connective tissue component of the normal bladder submucosa showing the aggregations of collagen fibers, fibroblastic cellular components and a small capillary. X 5,900.

Figure 8. Toluidine blue stained one micron section showing epithelial cell exfoliation and edema of the connective tissue component of the lamina propria in the bladder of the animal sacrificed seven hours following cyclophosphamide injection. X 640.

Fig. 5

Fig. 6

Figure 9. Hematoxylin and eosin stained section of the bladder epithelium in the twenty-four hour post injection animal. Note the denuded areas of mucosa along the lamina propria. X 640.

Figure 10. Toluidine blue stained one micron sections of bladder epithelium in the twenty-four hour animals. Note the areas of edema within the connective tissue component, occasional evidence of inflammatory cells and hemorrhage. X 640.

Figure 11. A marked area of hemorrhage and cellular degeneration in the animal sacrificed twenty-four hours following cyclophosphamide injection. Note the numerous irregularly shaped electron dense granules surrounded by a single or sometimes double membrane within and outside of degenerating polymorphonuclear neutrophils. X 11,800.

Figure 12. A degenerating polymorphonuclear neutrophil with granule expulsion. Note the similarities in size, shape and electron density to those granules described in Figure 11. X 11,200.

Fig. 9

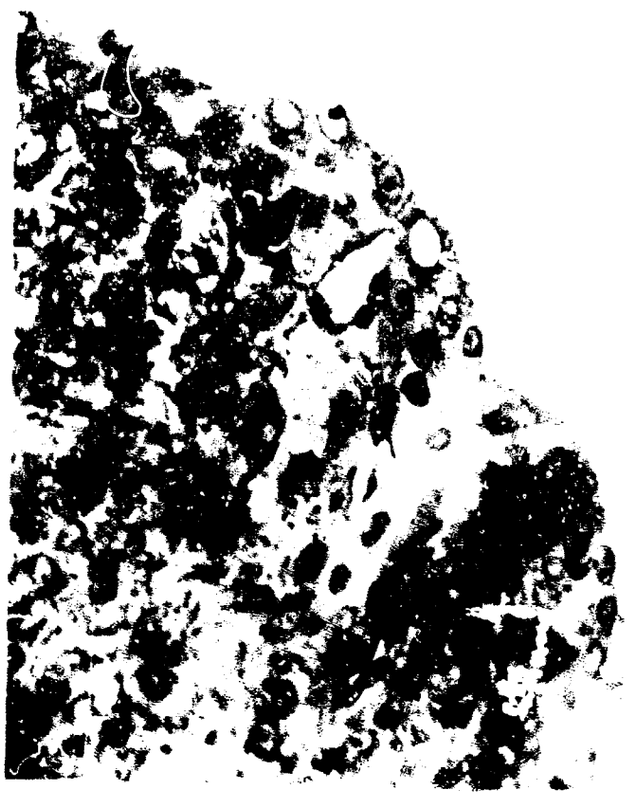


Fig. 10

Fig. 11

Fig. 12

Figure 13. Two transitional epithelial cells bordering the lumen in the bladder of the animal sacrificed at twenty-four hours. Note the large aggregations of lipid material and the small round vesicles near the luminal surface. Note also the long agranular elements of the endoplasmic reticulum and the occasional swelling of the cisternal elements. X 11,800.

Figure 14. Large uni-membranous lipid aggregations and the scattered fibrillar component of the cytoplasm are illustrated in this epithelial cell of the twenty-four hour animal. Note the dispersed ribosome aggregates, dense bodies, and swollen vesicular components of the endoplasmic reticulum. X 21,400.

Figure 15. Note the long strands of agranular endoplasmic reticulum with the occasional formation of whorls. Increase in the intercellular spaces is also illustrated. Twenty-four hour post injection animal. X 14,600.

Figure 16. Increase in the intercellular spaces along with ribosome associated endoplasmic reticulum is illustrated in these cells of the twenty-four hour animal. Evidence of the uni-membranous lipid deposits, degenerating mitochondria and numerous round vesicles are additionally illustrated. X 20,400.

Fig. 13

Fig. 14

Fig. 15

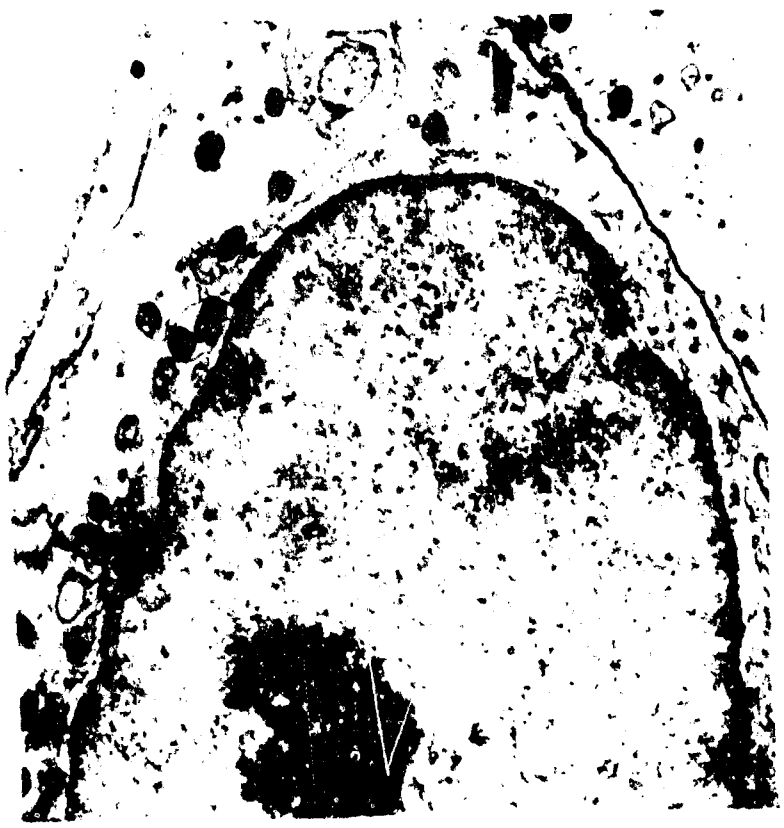


Fig. 16

Figure 17. Cells of the twenty-four hour animal possessing numerous round vesicles, lipid deposits and a moderate degree of swelling of the vesicular element of the Golgi apparatus. X 11,900.

Figure 18. Damaged and degenerating mitochondria along with dispersed ribosome aggregations are present within the cytoplasm of cells in the twenty-four hour animal. X 38,300.

Figure 19. Occasional vesicles of the compressed type were seen in some of the cells in the twenty-four hour injection animal. Note the irregularly shaped dense bodies and swollen vesicular components of the agranular endoplasmic reticulum. X 14,800.

Figure 20. Higher magnification of the cytoplasmic component illustrating numerous irregularly shaped round vesicles, dense bodies and swollen vesicular components of the endoplasmic reticulum. Note the large focal area of cytoplasmic degeneration. Twenty-four hour post injection animal. X 21,400.

Fig. 17

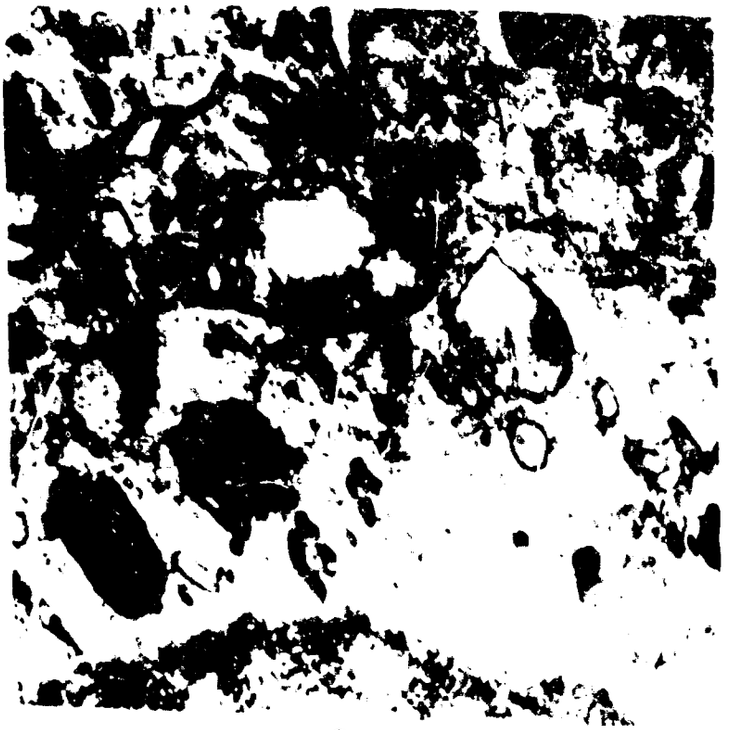


Fig. 18

Fig. 19

Fig 20

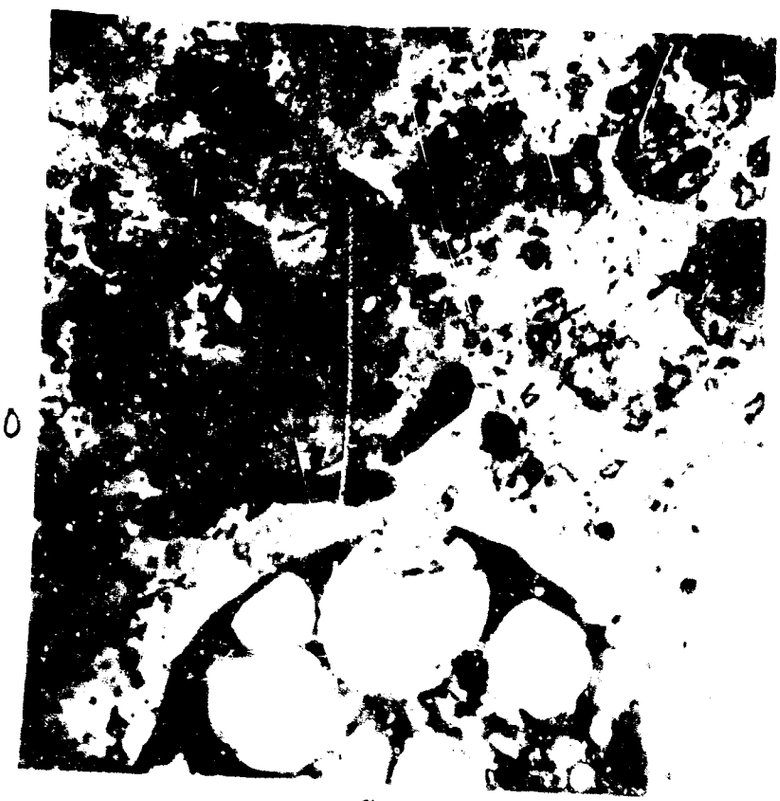


Figure 21. Hematoxylin and eosin stained bladder epithelium of the animal sacrificed four days post injection illustrating edema and inflammatory reaction in the bladder mucosa and submucosa. X 640.

Figure 22. Toluidine blue stained one micron sections illustrating the large inter and extracellular spaces between the epithelial cells in the four day animal. Note the numerous erythrocytes and the moderately large dark intracytoplasmic bodies. X 640.

Figure 23 Electron microscopically the mucosal bladder cells appear disorganized due to the numerous extracellular spaces which are filled with erythrocytes. Note the numerous cytoplasmic "foot processes" present in the intercellular spaces. Four day post injection animal. X 3,400.

Figure 24. Evidence of multinucleation was evident in some of the bladder mucosal cells of the four day animal. Note the intercellular spaces and the microvilli projections into the extracellular spaces. X 3,400.

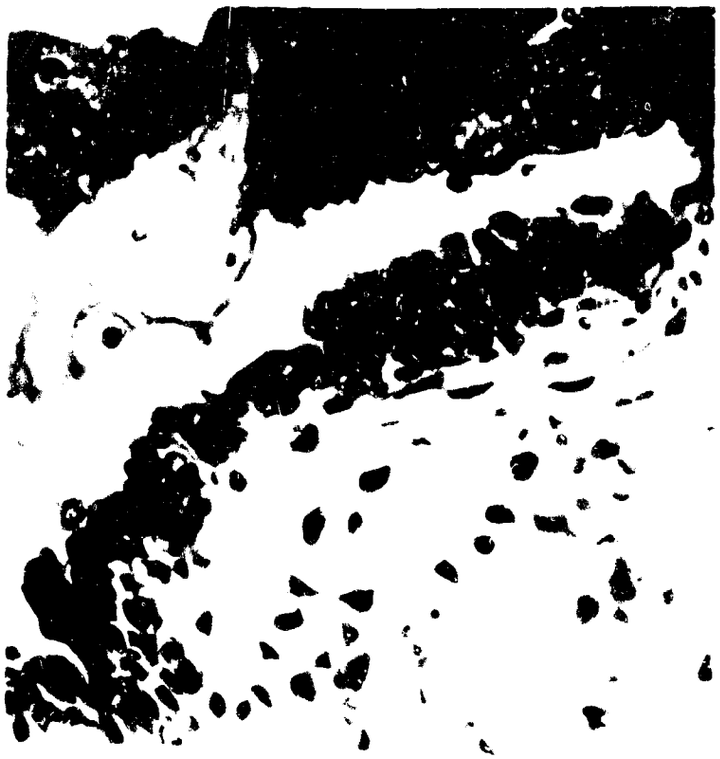


Fig. 21

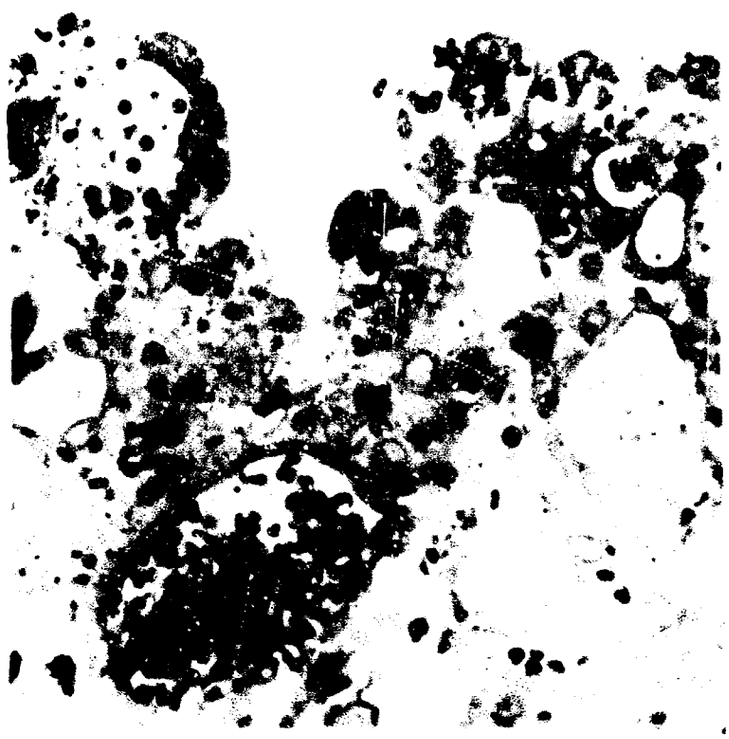


Fig. 22

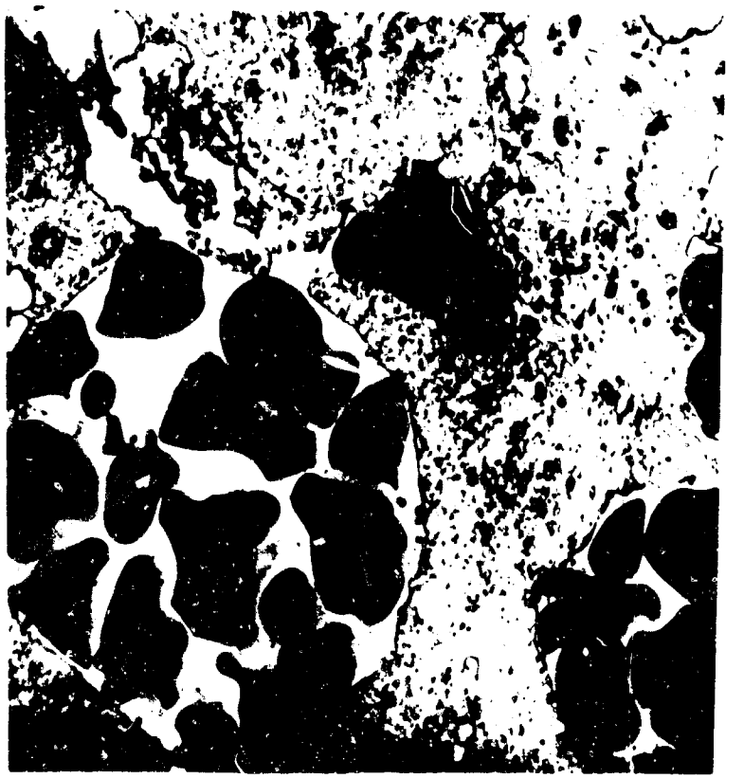


Fig. 23

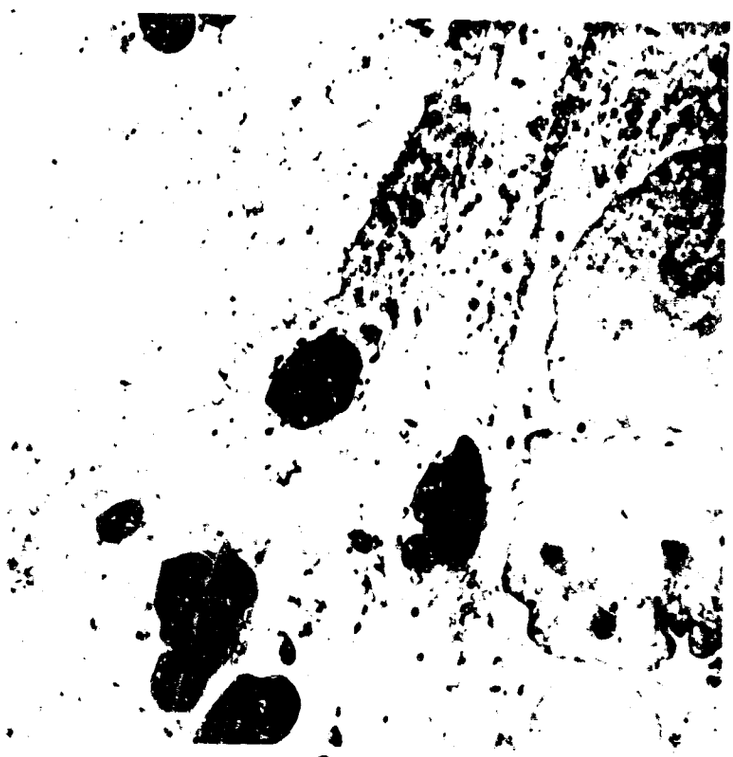


Fig 24

Figure 25. Noticeable within some animals in this four day group were cytoplasmic aggregations of amorphous appearing material. Note the creation of the intercellular spaces which appear above and below the areas of the desmosomes or terminal bars. X 4,000.

Figure 26. The intercellular spaces (IS) appear along the opposing cell membranes of the plasma membrane and end at the area of the terminal bar proximal to the lumen. Note the degenerated mitochondria, numerous small round vesicles and the small elements of the endoplasmic reticulum. Four day post injection animal. X 5,900.

Figure 27. Evidence of cellular exfoliation and cell death are illustrated through the lack of cellular cohesion, nuclear atrophy and mitochondrial degeneration. Four day post injection animal. X 4,400.

Figure 28. Small elements of the endoplasmic reticulum, isolated aggregates of amorphous material and dense bodies are illustrated in some of the cells of the bladder epithelium of the animals sacrificed four days post injection. X 6,000.

Fig. 25

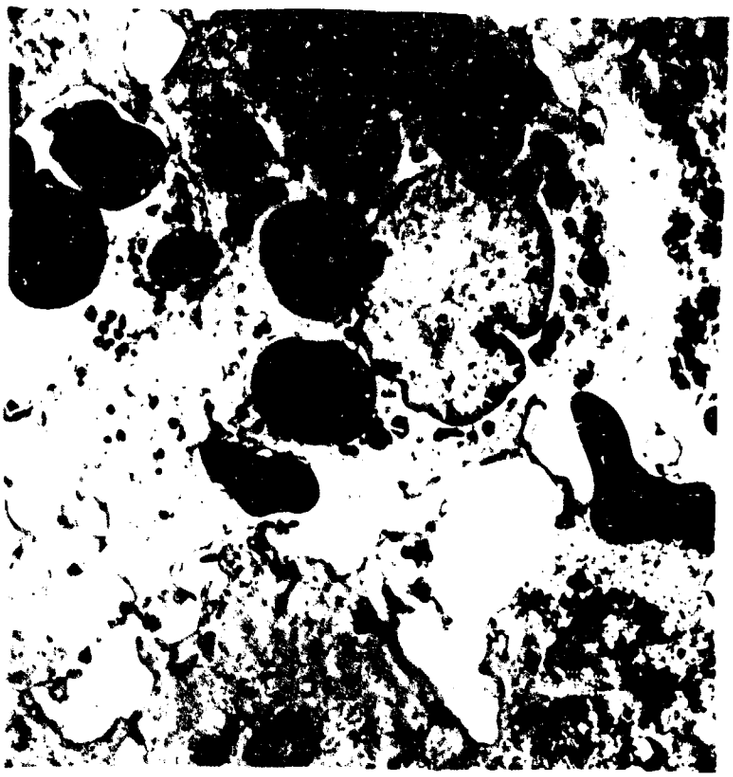


Fig. 26

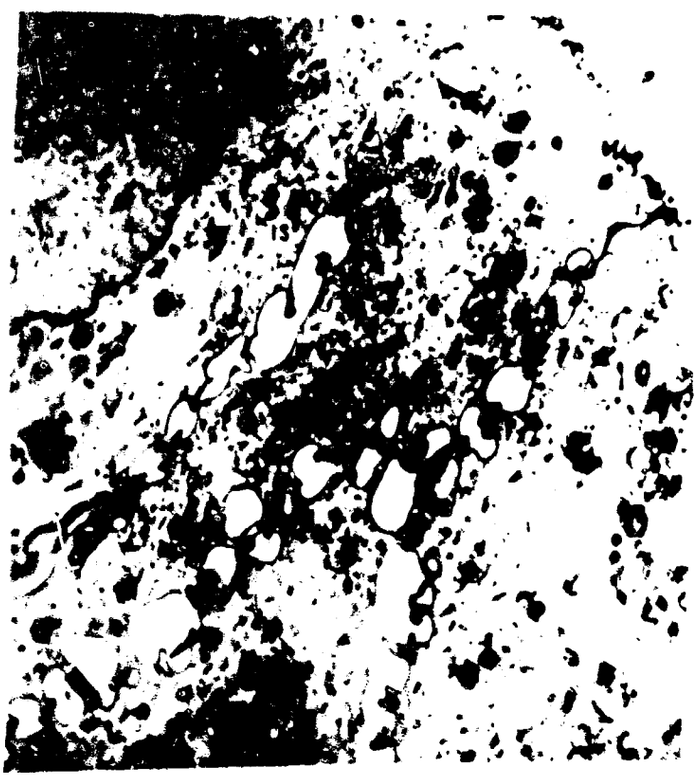


Fig. 27

Fig. 28

Figure 28. Whorls of agranular membranes surrounding focal cytoplasmic areas are illustrated. Note the numerous filaments of rough surfaced endoplasmic reticulum. Four day post injection animal. X 5,900.

Figure 30. Suggested binucleation with cytoplasmic edema, dense bodies and swelling of the vesicular components of the endoplasmic reticulum is illustrated. Note the microvilli projections into the intercellular space. Four day post injection animal. X 7,000.

Figure 31. Binucleation with alterations in the shape of the nuclei and increase in nuclear chromatin content is evidenced within some of the transitional cells of the animals sacrificed four days post injection. Note the numerous small elements of the rough surfaced endoplasmic reticulum and the relatively large dense granules. X 7,400.

Figure 32. Numerous prominent Golgi areas along with elements of rough surfaced endoplasmic reticulum are illustrated. Four day post injection animal. X 7,400.

Fig. 29

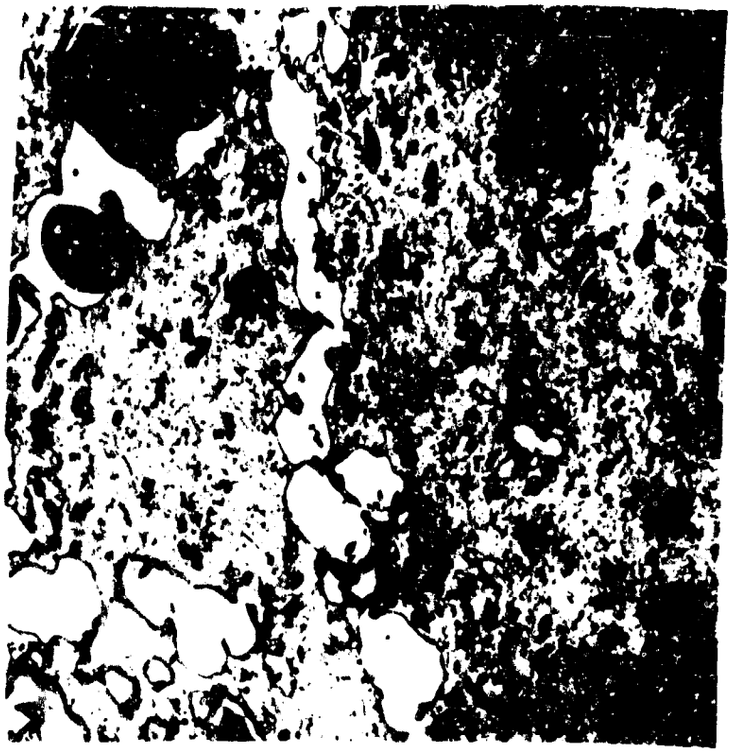
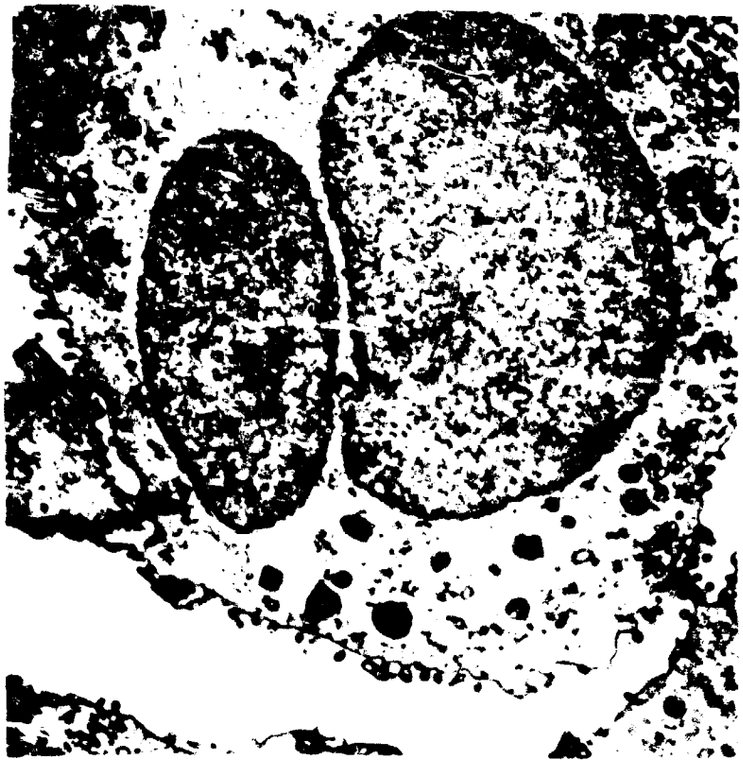


Fig. 30



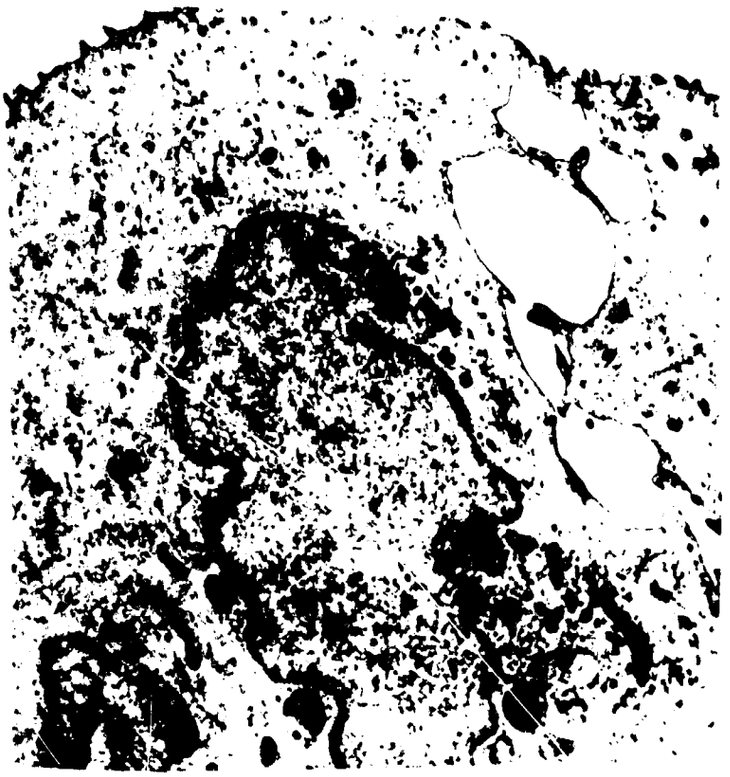


Fig. 31

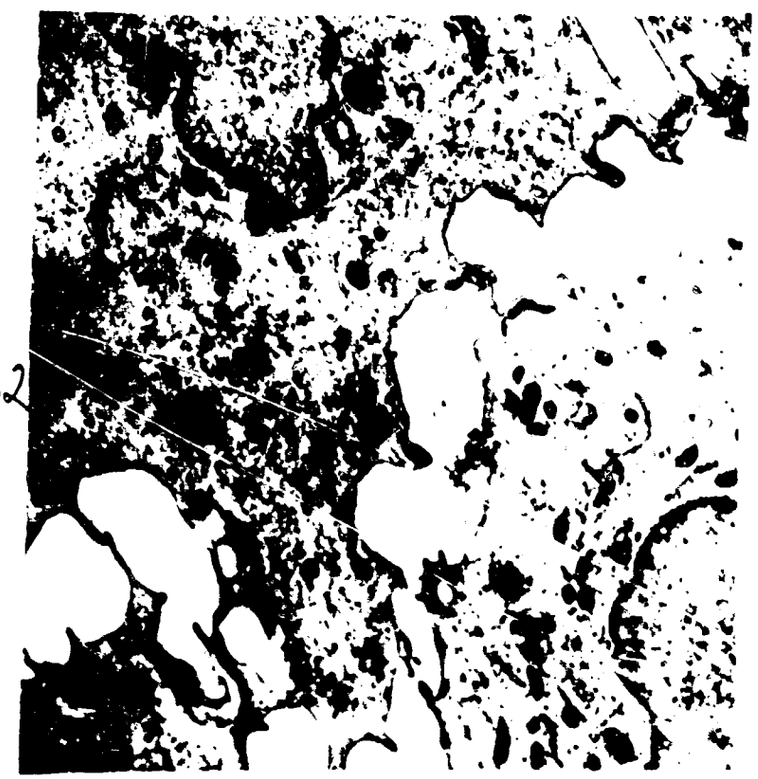


Fig. 32

Figure 33. Numerous small round vesicles are located near the luminal surface of some of the transitional cells in this four day post injection group. Note the prominent nucleoli, dense bodies and Golgi areas. X 10,600.

Figure 34. The fibroblastic cellular component of the connective tissue shows marked enlargement in the cisternal spaces of the endoplasmic reticulum with the presence of an amorphous, electron dense material within the cisternae. The large cell with prominent nucleus most probably represents an endothelial cell of a vascular component. Four days post injection animal X 5,900.

Figure 35. Toluidine blue one micron sections of the multilayered dysplastic epithelium of the eight day post injection animal is illustrated. X 640.

Figure 36. Marked cellular degeneration is illustrated in some of the cells of the eight day animals with disappearance of the plasma membrane and peripheral dispersion of the nuclear chromatin. X 5,900.

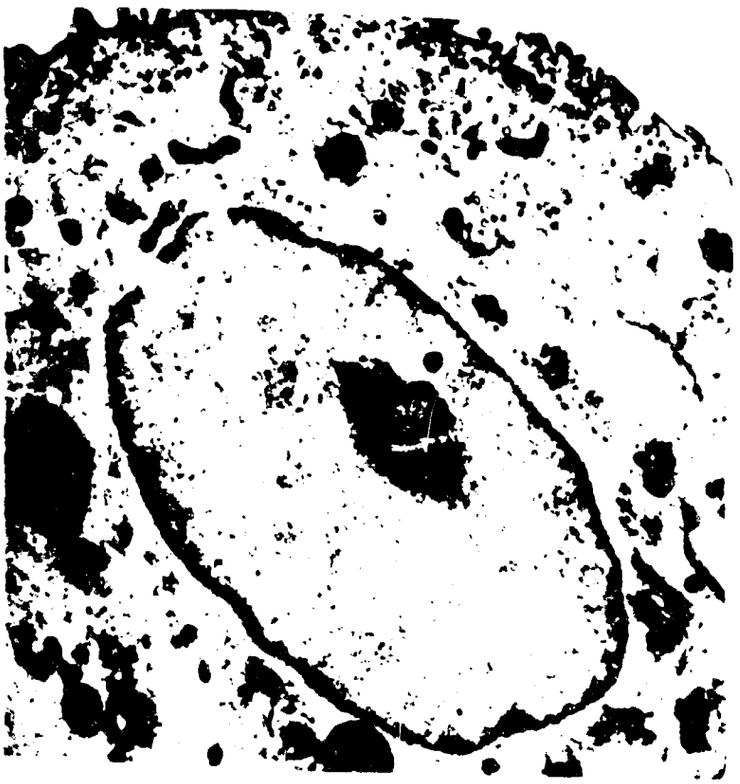


Fig. 33

Fig. 34

Fig. 35

Fig. 36

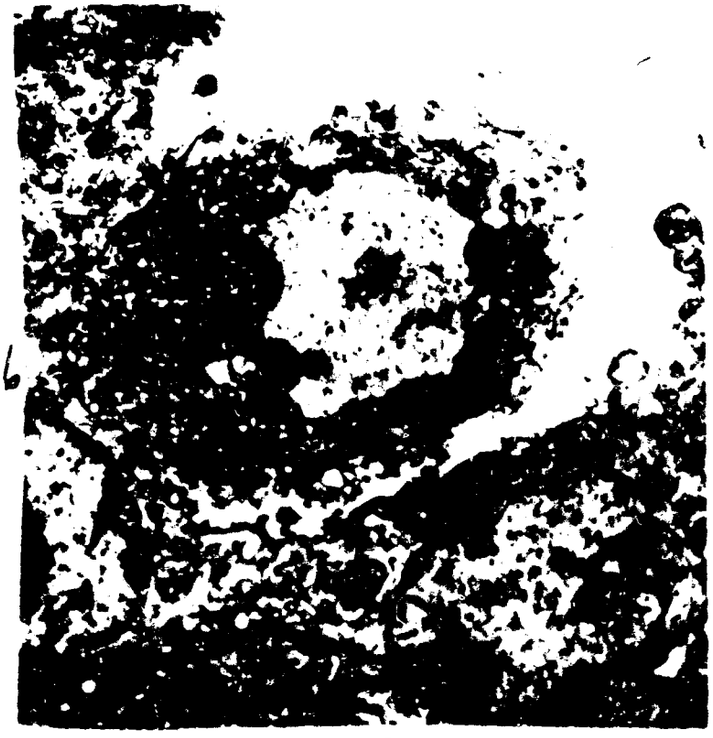


Figure 37. Higher magnification of the same cell illustrated in Figure 36. Note the numerous round granules surrounded by a single unit membrane and the almost complete dissolution of portions of the nuclear membrane. Eight day post injection animal. X 7,400.

Figure 38. Areas of altered mitochondria are illustrated in some of the bladder transitional cells of the eight day post injection animal. Note the prominent desmosomes and adjoining tonofibrils and the numerous irregularly shaped round vesicles. X 9,700.

Figure 39. Evidence of mitochondria alteration with deposits of small quantities of electron dense material within the mitochondria matrix is illustrated. Note the unequal distribution of the fine fibrillar component within the cytoplasm. Eight days post injection animal. X 11,000.

Figure 40. Numerous irregularly shaped vesicles some suggesting the sequestration of cytoplasmic components are illustrated. Note the prominent desmosome (D) with adjoining tonofibrils. Eight days post injection animal. X 15,300.

Fig. 37

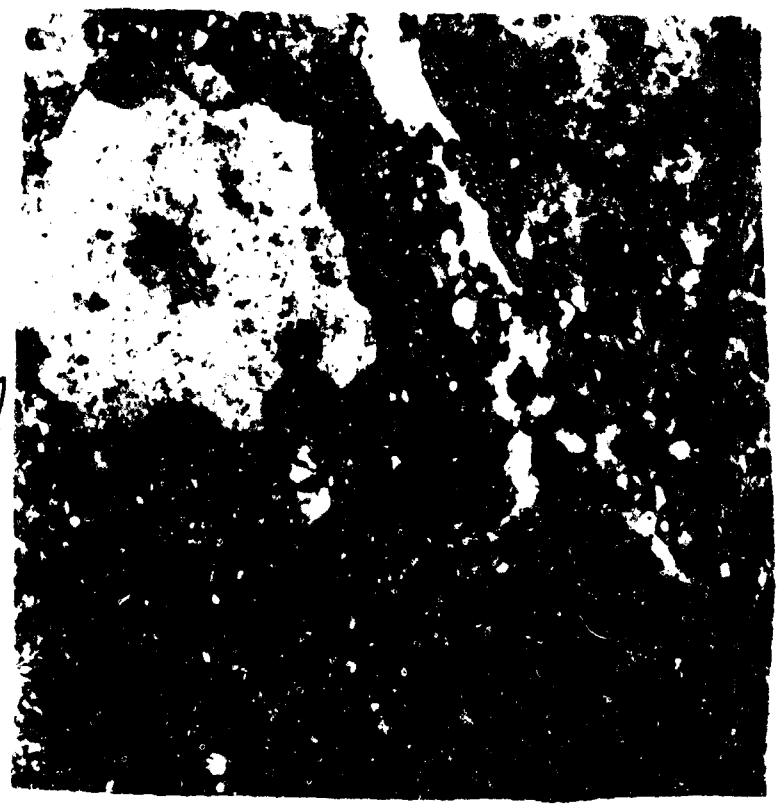


Fig. 38

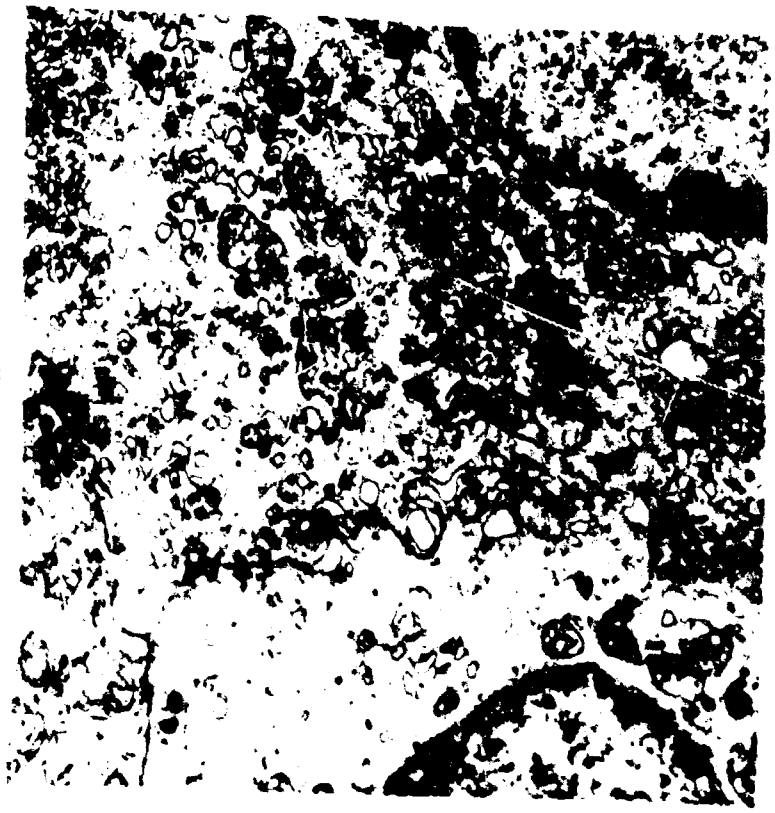


Fig. 39

Fig. 40

Figure 41. Whorls of smooth membranes surrounding focal cytoplasmic components were evidenced in some cells of the eight day animals. Note the altered mitochondria, dense bodies and large intercellular spaces. X 7,400.

Figure 42. Prominent in some cells of the eight day animals were the prominent Golgi zones (G). Note also the long filaments of rough surfaced endoplasmic reticulum. Eight days post injection animal. X 7,400.

Figure 43. The cells of the multilayered epithelium show nuclei with some irregularities in shape and occasionally demonstrate peripheral aggregations of nuclear chromatin. Eight days post injection animal. X 5,400.

Figure 44. Within the cells of this multilayered epithelium are large extensive filaments of rough surfaced endoplasmic reticulum and prominent Golgi zones. Note the interdigitating cytoplasmic "foot processes". Eight days post injection animal. X 7,400.

Fig. 41

Fig. 42

Fig. 43

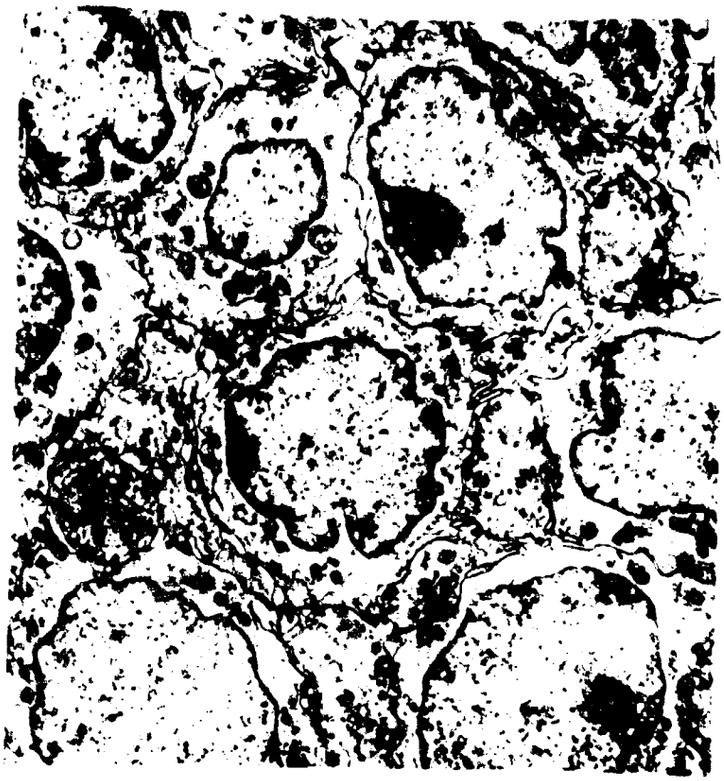


Fig. 44

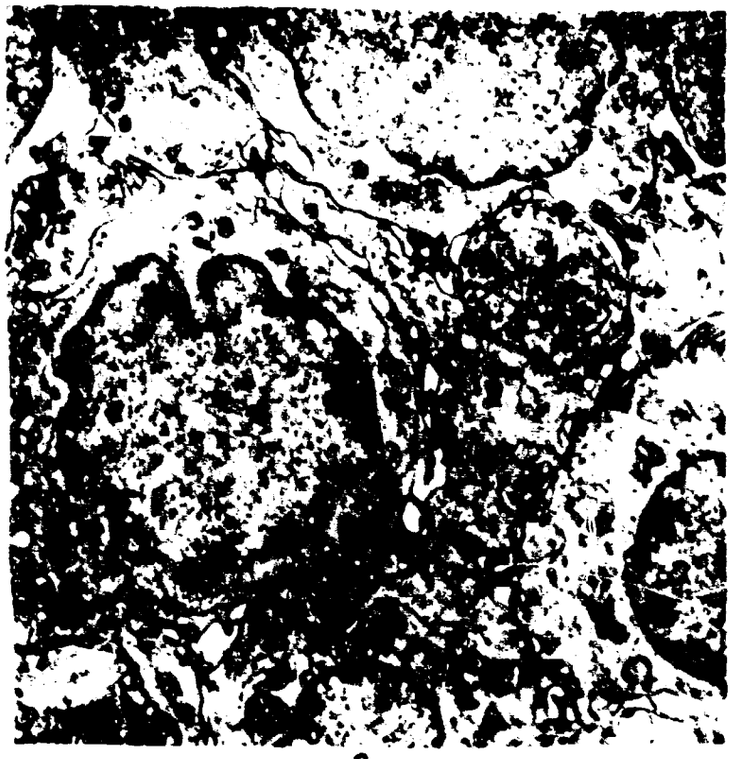


Figure 45. The cells of the liver in the nine day post injection animals showed evidence of cloudy swelling with no fatty metamorphosis. X.640.

Figure 46. The kidneys of the nine day animals showed evidence of tubular damage with occasional erythrocytes within the tubules. X 640.

Figure 47. The testicle in these animals show a marked degree of hemorrhagic necrosis. Nine day post injection animal. X 640.

Figure 48. The bladder shows a slight amount of edema, no hemorrhage and questionably normal, multilayered epithelium. Nine day post injection animal. X 640.

Fig. 45

Fig 46

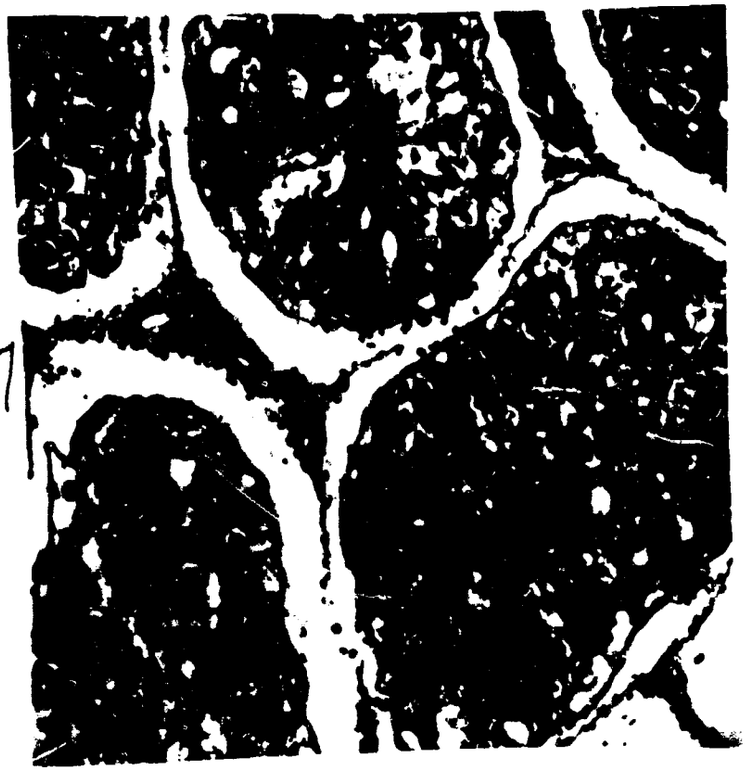


Fig. 47

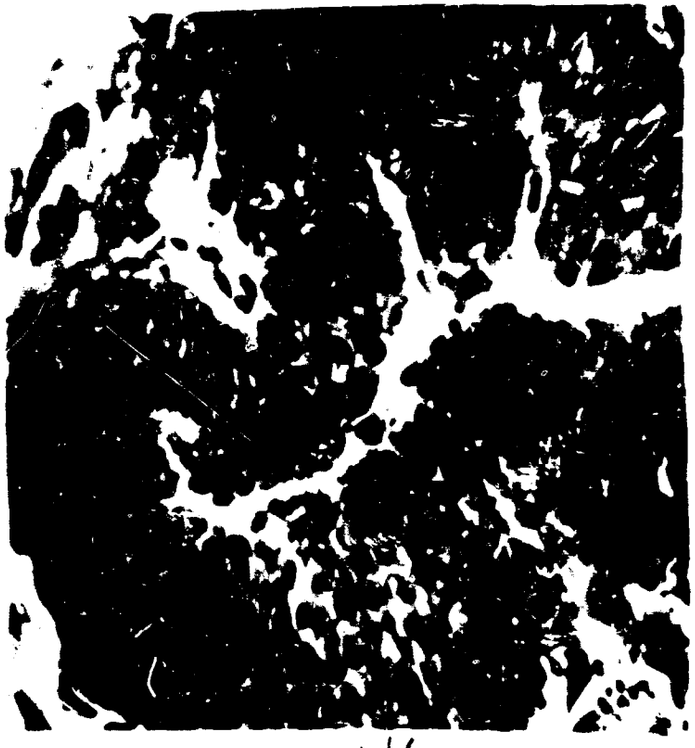


Fig. 48

Figure 49. A transitional epithelial cell in the nine day animals illustrating cytoplasmic vacuolization. Note the multiple prominent nucleoli and binucleation. X 6,700.

Figure 50. An area within a transitional epithelial cell bordering the lumen shows the formation of a large cytoplasmic vacuole. Note other smaller cytoplasmic vacuoles along with the occasional microvilli protrusion into the lumen area. Nine days post injection animal. X 7,400.

Figure 51. Within other transitional cells of the nine day animals were areas of cytoplasmic sequestration of degenerating components. Note the nucleus of the cell with multiple nucleoli and peripheral nuclear chromatin aggregations along with frequent filaments of rough surfaced endoplasmic reticulum within the cytoplasm. X 7,400.

Figure 52. Note the large membrane bound sequestered cytoplasmic area in which are found numerous degenerating cellular organelles. In addition, small vesicles of the compressed vesicle configuration are present within the cytoplasmic component of some of these cells. Nine days post injection animal. X 5,900.

Fig 49

Fig. 50

Fig. 51

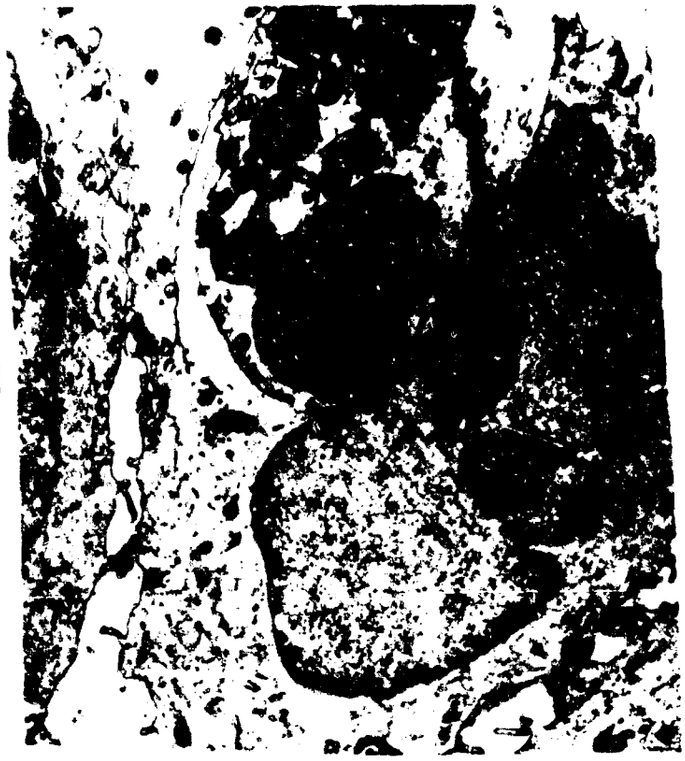


Fig. 52

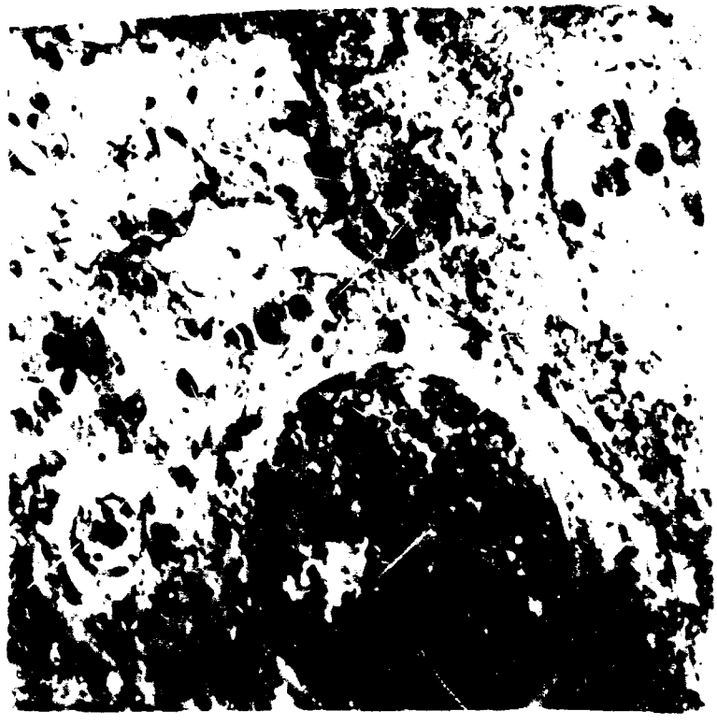


Figure 53. A transitional cell bordering the lumen with large nuclear vacuole is illustrated. Cytoplasmic vacuoles and microvilli protrusions into the luminal area are present. Nine day post injection animal. X 6500.

Figure 54. Mitochondria within the cytoplasmic component of a cell illustrating marked alteration and evidence of degeneration. Nine days post injection animal. X 10,700.

Figure 55. Numerous filaments of rough surfaced endoplasmic reticulum and prominent Golgi zones characterized some cells of the nine day post injection animals. Note the numerous round vesicles, and dense bodies. X 11,800

Figure 56. A cytoplasmic area within one cell illustrating numerous dense granules and degenerating cellular organelles. Note areas within adjacent cells which contain filaments of rough surfaced endoplasmic reticulum. Nine day post injection animal. X 10,700.

Fig. 53

Fig. 54

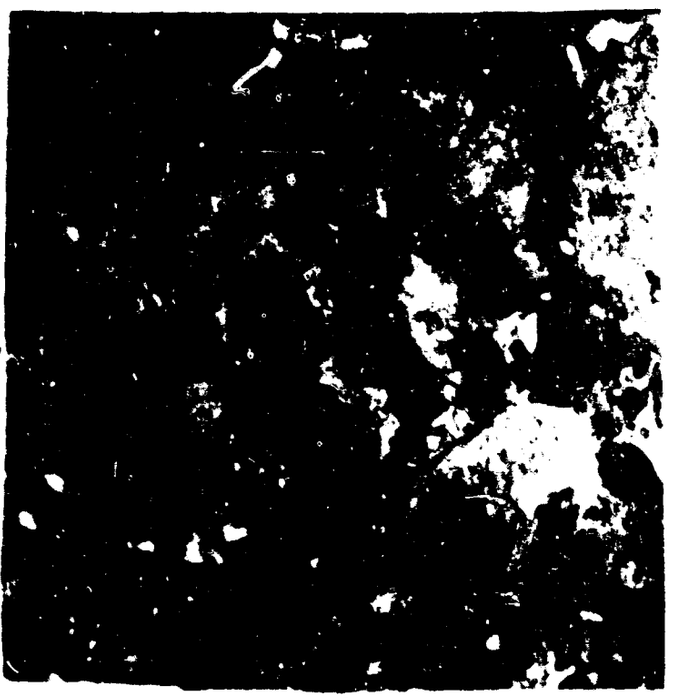


Fig. 55

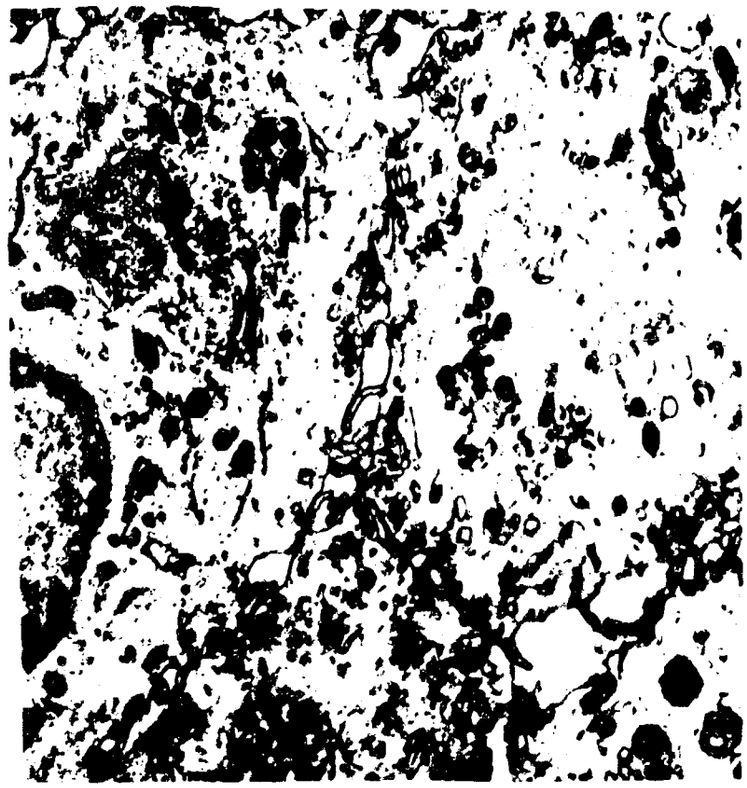


Fig. 52

Figure 57. A markedly altered fibroblast within the connective tissue component is illustrated. Note the numerous cytoplasmic vesicles. Nine day post injection animal. X 5,900.

Figure 58. Evidence of some cellular exfoliation with degeneration of the cells is illustrated. Numerous small round vesicles, most probably lysosomes, are found within the one cell. Nine day post injection animal. X 12,300

Figure 59. Evidence of cellular exfoliation proceeded in some areas as far down as the basement membrane. Note the one cell which is almost completely degenerated. Nine day post injection animal. X 5,900.

Figure 60. A cell bordering the lumen in the nine day post injection animal illustrating vacuole inclusions within the cytoplasm. Note large dense body and elements of the rough surfaced endoplasmic reticulum. X 7,400.

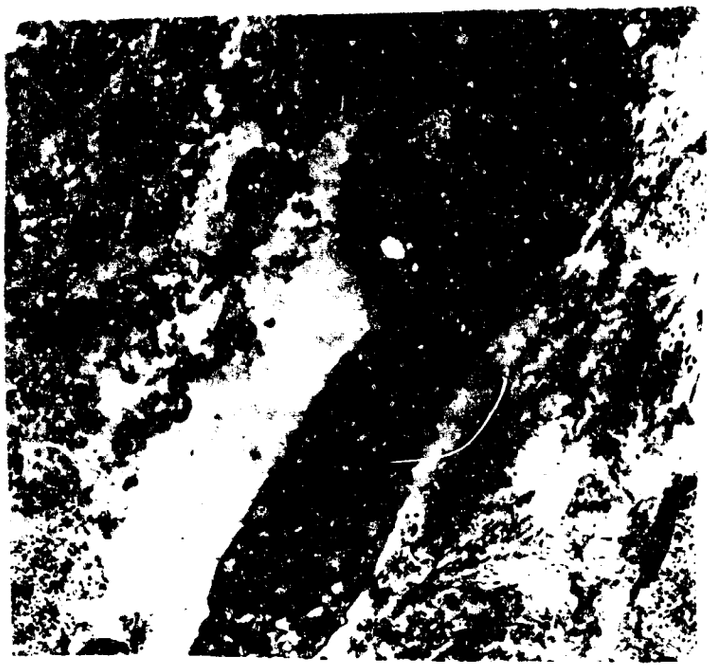


Fig. 57

Fig. 58

Fig. 59

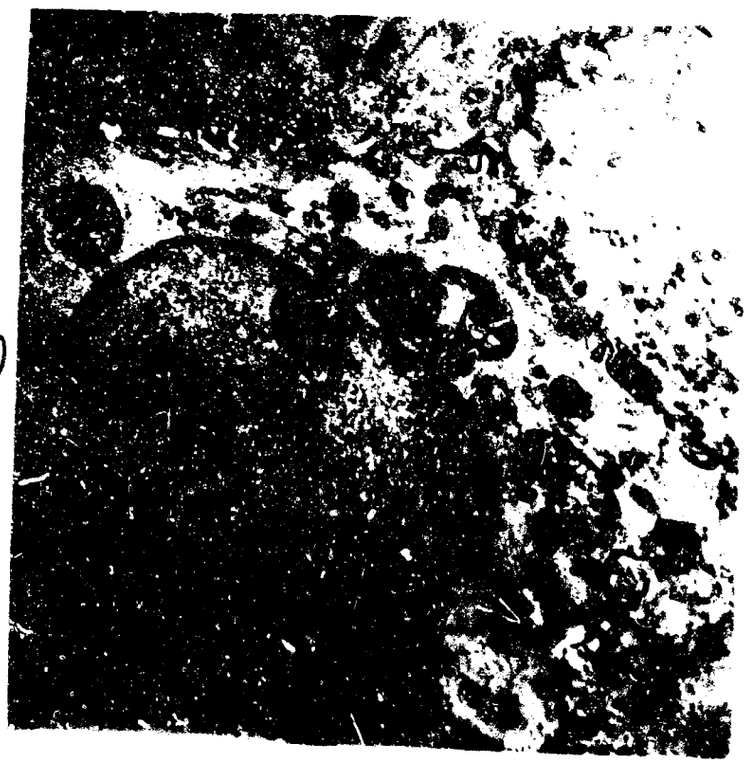


Fig. 60

Figure 61. Transitional epithelial cells from the nine day post injection animal illustrating cytoplasmic vacuole inclusions of varying sizes, dense bodies, rough surfaced endoplasmic reticulum and evidence of nuclear atrophy. X 5,900.

Figure 62. Within the area of the multilayered epithelium in the nine day post injection animal were cells in which the nuclei appeared somewhat irregular in shape with occasional multiple nucleoli and peripheral aggregations of nuclear chromatin. Note the one area adjacent to these active cells with numerous dense bodies and cytoplasmic vacuoles. X 4,400.

Figure 63. Cells of the multilayered epithelium of the nine day post injection animal. Note the cytoplasmic foot processes and strands of the rough surfaced endoplasmic reticulum. X 5,900

Figure 64. Cells of the multilayered epithelium at the area of the basement membrane. Note the long filaments of rough surfaced endoplasmic reticulum and the intercellular spaces. Nine day post injection animal. X 6,800

Fig. 61

Fig. 62

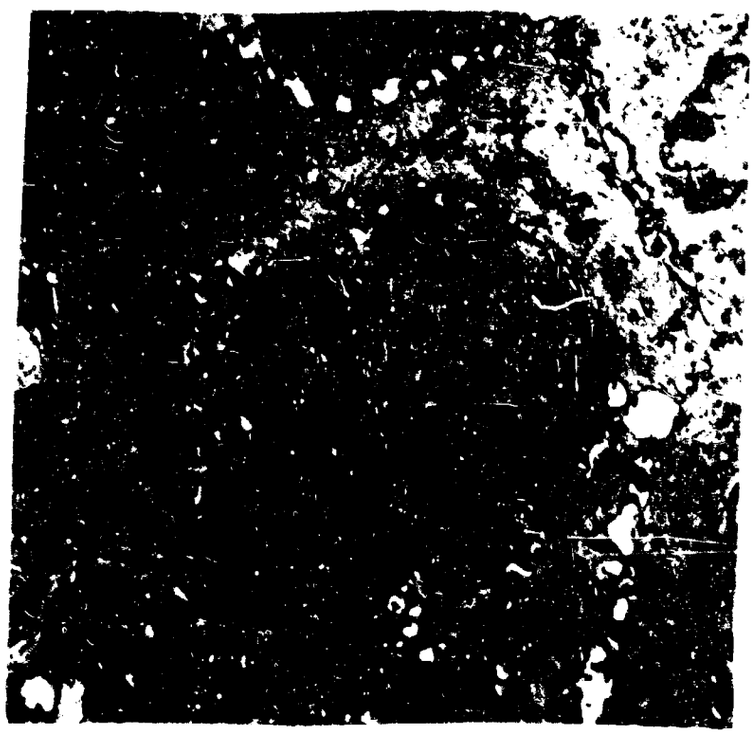


Fig. 63

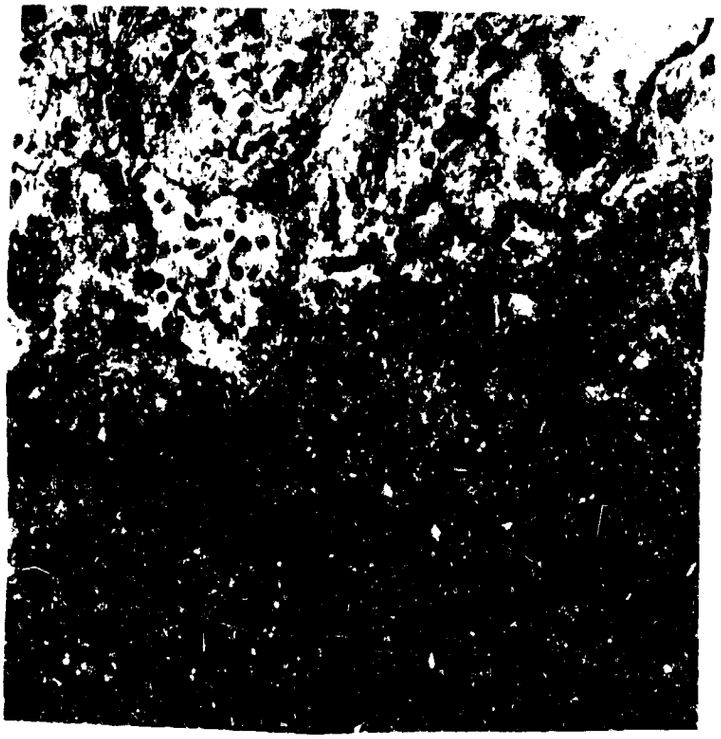


Fig. 64

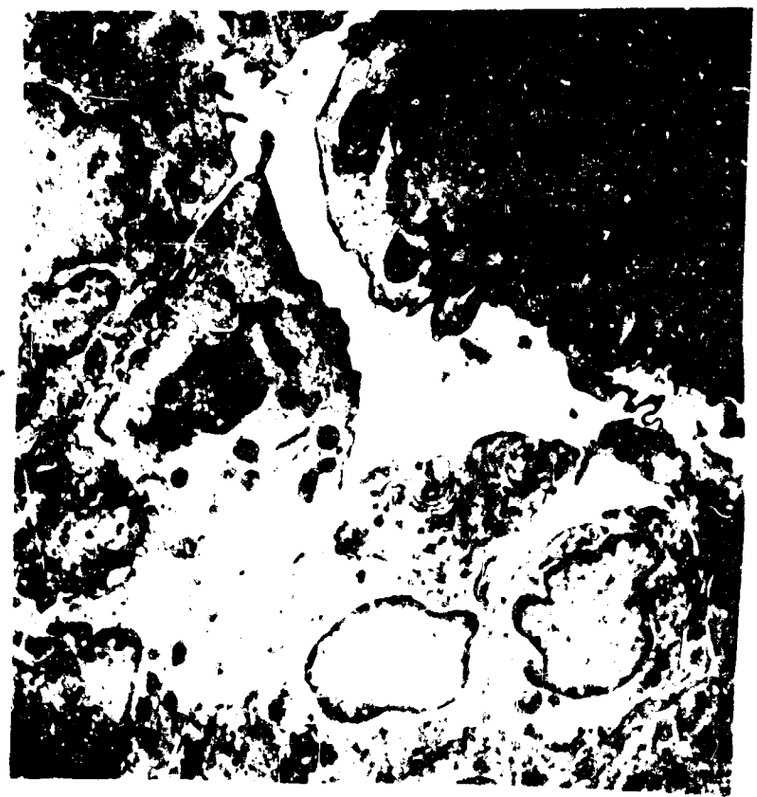


Figure 65. Focal areas of tubular damage within the kidneys of the ten day post injection animal. X 640.

Figure 66. Toluidine blue stained one micron section of the bladder transitional epithelium in the animal sacrificed ten days following cyclophosphamide injection. Note the amorphous material between some of the cells of this multilayered epithelium. X 1,600.

Figure 67. Within the multilayered epithelium of the ten day post injection animals were the continuing presence of the intercellular spaces. Note the prominent and multiple numbers of desmosomes along areas of the plasma membrane. X 5,900.

Figure 68. Other areas within this multilayered epithelium there was evidenceof filling within the intercellular spaces. Ten days post injection animal. X 6,500.

Fig. 65

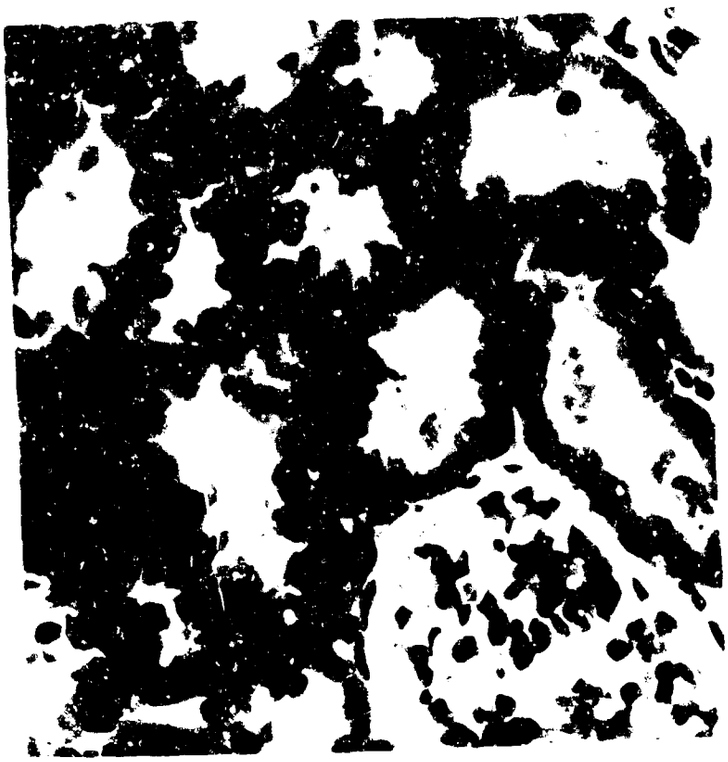


Fig. 66

Fig. 67

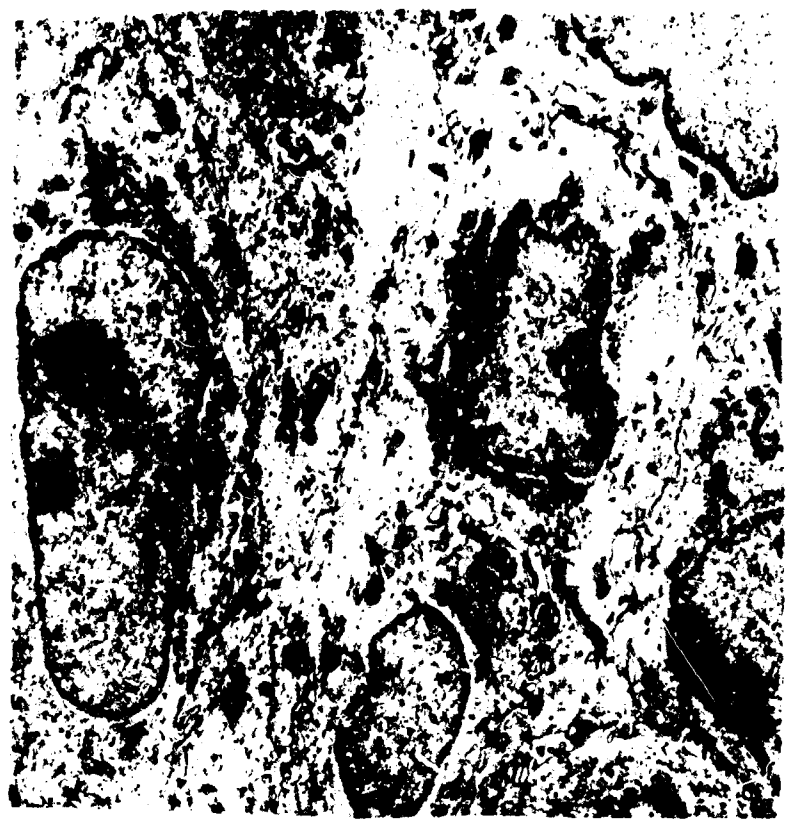


Fig. 68

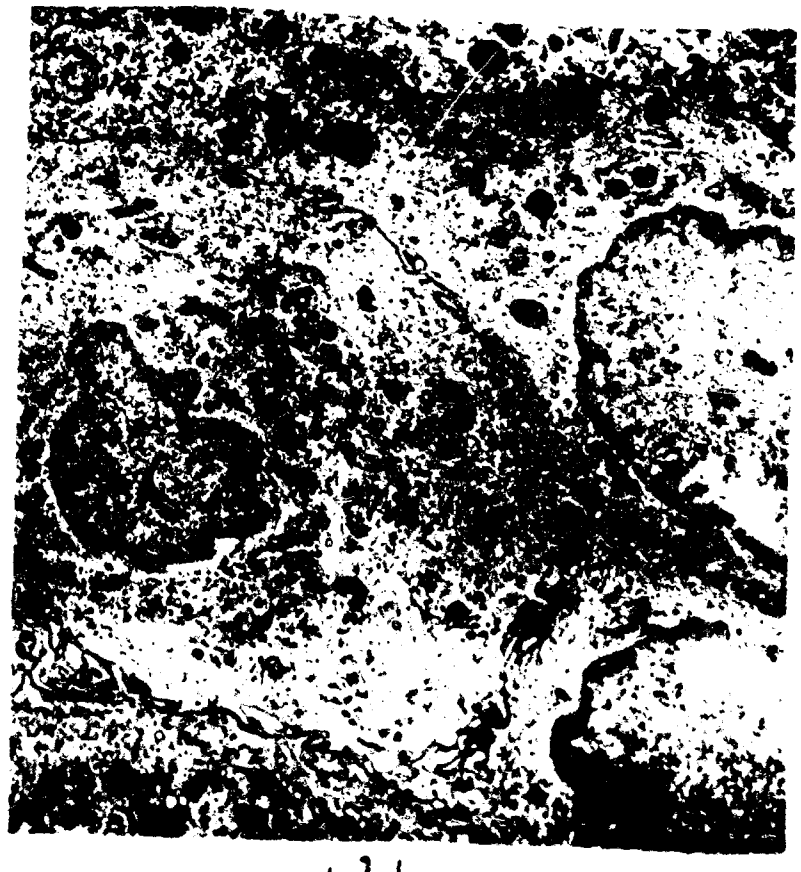


Figure 69. The fine fibrillar component within the cytoplasm appears in the cell to be aligned along the area of the intercellular spaces of the plasma membrane. Note the multiple nucleoli and electron denseness of the nuclear chromatin. Ten days post injection animal. X 5,900.

Figure 70. Areas within the intercellular spaces of these cells appear to be partially filled with an amorphous electron dense material. Note the numerous elements of the rough surfaced endoplasmic reticulum. Ten day post injection animal. X 7,400.

Figure 71. Evidence of cytoplasmic vacuoles, dense bodies and numerous round vesicles are illustrated in some of the cells. Note the appearance of the electron dense material within the intercellular spaces of some of the cells. Ten days post injection animal. X 3,400.

Figure 72. Higher magnification of one of the cells of this multi-layered epithelium illustrating the electron dense material within the intercellular spaces. Note the numerous round vesicles near the plasma membrane and the multiple elements of rough surfaced endoplasmic reticulum. Ten day post injection animal. X 7,400.

Fig. 69

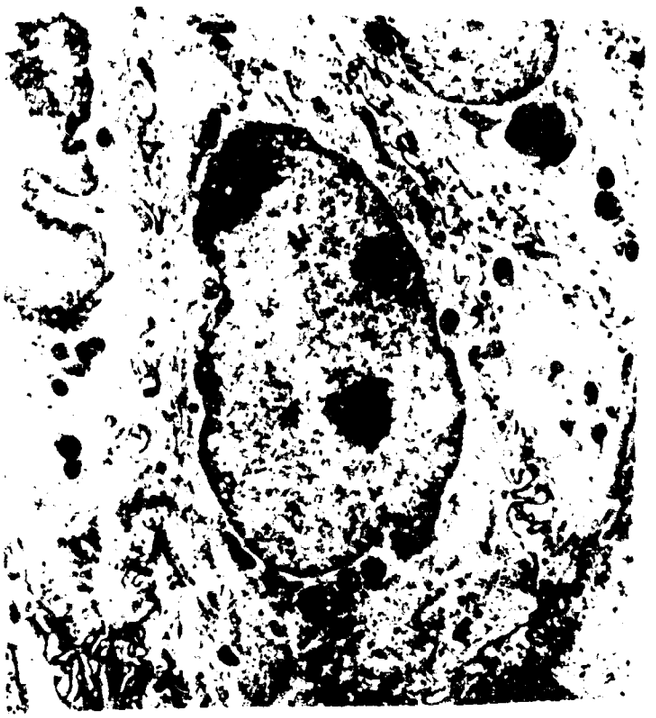


Fig. 70

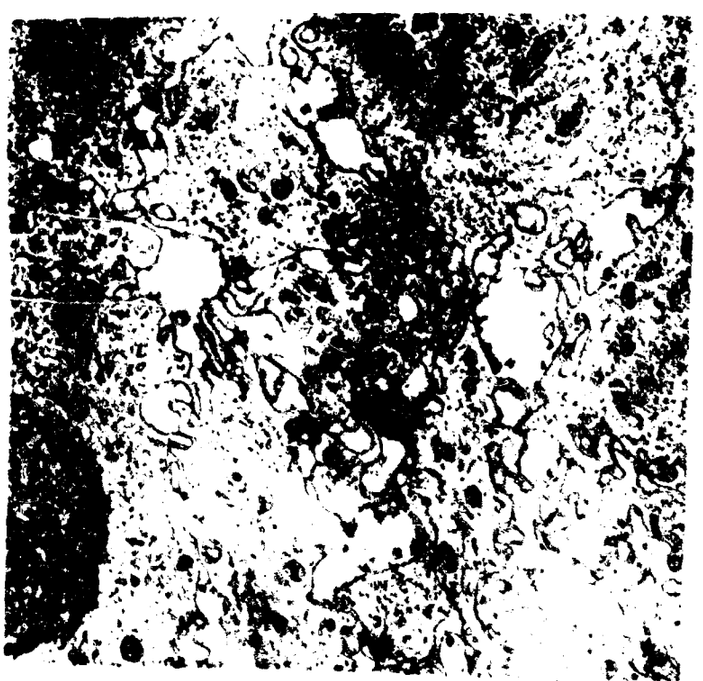


Fig. 71

Fig. 72

Figure 73. The intercellular filling with the electron dense material occurs periodically along the plasma membrane. In some areas there appears to be communication between individual cells and the electron dense material within the intercellular spaces. Note the prominent desmosome with adjoining tonofibrils, the polyribosomal aggregations and elements of the rough surfaced endoplasmic reticulum. Ten days post injection animal. X 15,300.

Figure 74. Evidence of cellular degeneration with swelling of the endoplasmic reticulum, numerous round vesicles, dense bodies and aggregates of amorphous electron dense materials are illustrated within areas of this multilayered epithelium. Ten day post injection animal. X 7,400.

Figure 75. Alterations in the mitochondria and swelling of some of the components of the endoplasmic reticulum are illustrated. Note numerous dense bodies of varying sizes and shapes along with densely packed intracytoplasmic fibers and large intercellular spaces. Ten day post injection animal. X 9,200.

Figure 76. An area of cytoplasmic sequestration is illustrated with various organelle components and lipid accumulations. Ten day post injection animal. X 5,900.

Fig. 13

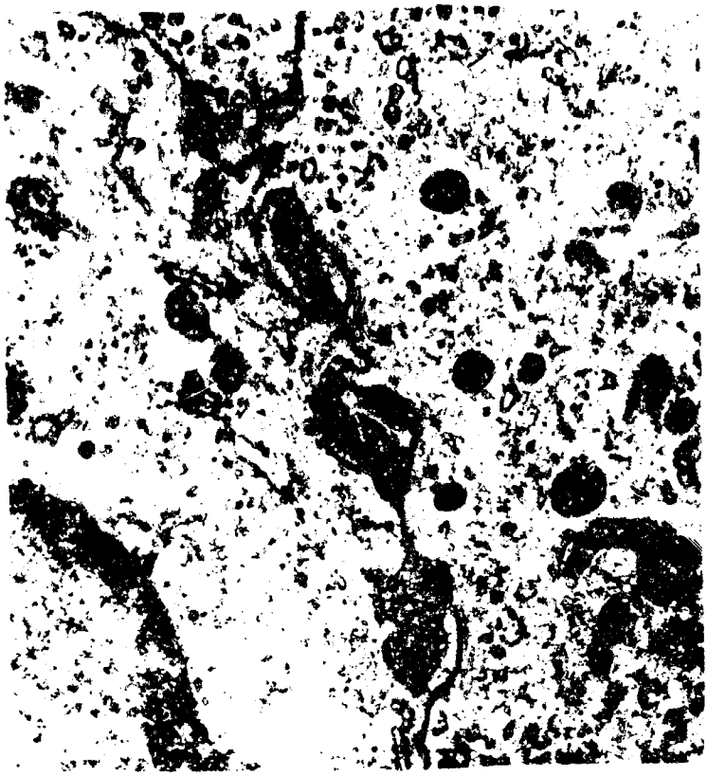


Fig. 14

Fig. 75

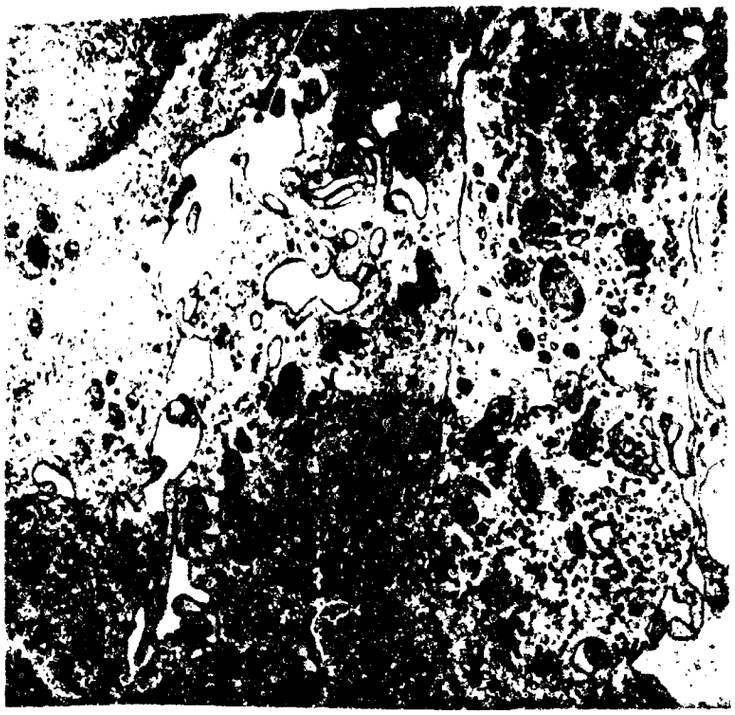


Fig. 76

Figure 77. Transitional epithelial of the ten day animal illustrating prominent Golgi zones. Note numerous dense granules and small focally sequestered cytoplasmic components. X 7,400.

Figure 78. Note the large Golgi zone within this transitional cell of the ten day animal. Polyribosomal aggregations, rough surfaced elements of the endoplasmic reticulum are also illustrated. X 10,600.

Figure 79. Evidence of intercellular filling near the cells lining the lumen is illustrated. Note numerous round vesicles, some containing extremely electron dense material, and the microvilli projections of the surface cell. Ten day post injection animal. X 5,900.

Figure 80. Higher magnification of the surface cell in this ten day animal showing the microvilli protrusions into the luminal space. X 15,300.

Fig. 77

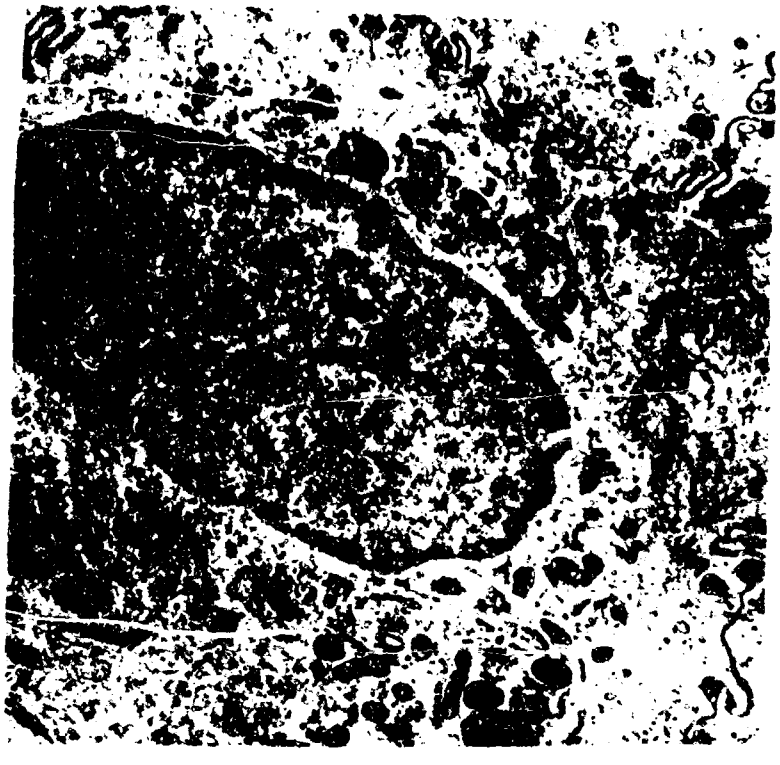


Fig. 78

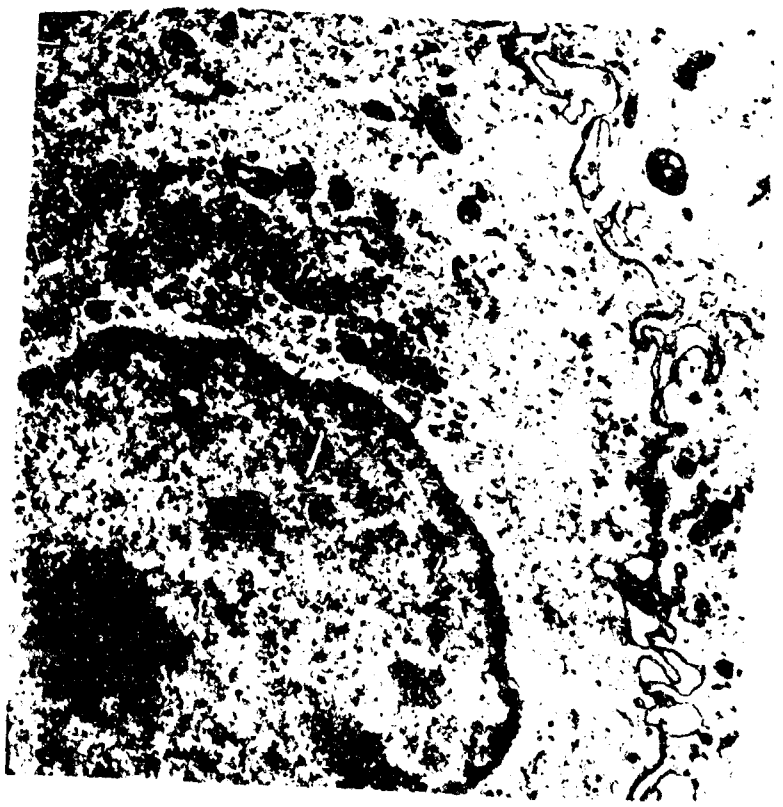


Fig. 79

Fig. 80

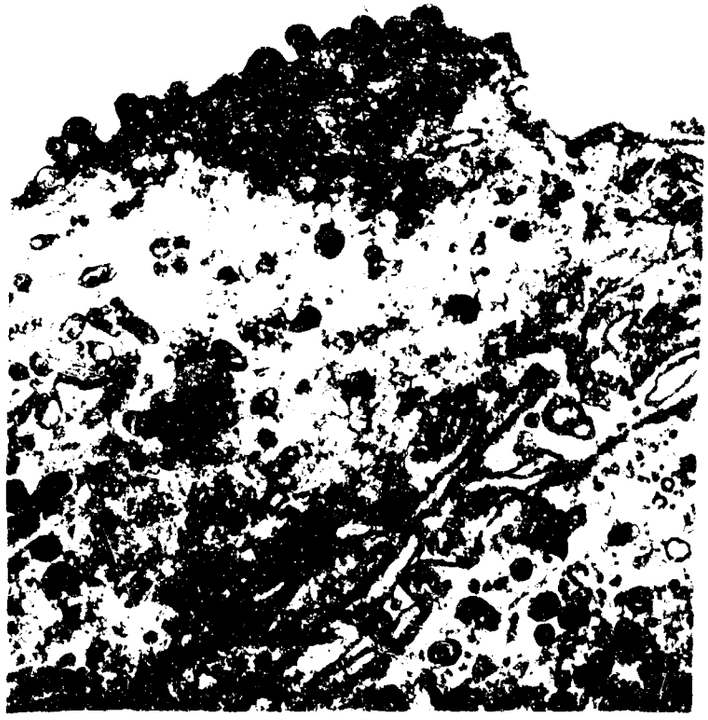


Figure 81. Evidence of the amorphous electron dense material within the connective tissue component of the bladder submucosa. Ten day post injection animal. X 4,000.

Figure 82. Evidence of the amorphous material along the basement membrane of the epithelial layer. Note the apparent communication between the area of the basement membrane and the intercellular spaces. Ten day post injection animal. X 15,300.

Figure 83. Toluidine blue stained one micron section showing an area of the regenerating multilayered bladder epithelium of the thirteen day post injection animal. Note the numerous multiple nucleoli present in many of these cells. X 640.

Figure 84. Numerous cells with prominent nuclei are illustrated as representative of the multilayered epithelium. Note the numerous cytoplasmic organelles. Thirteen day post injection animal. X 3,400.

Fig. 81

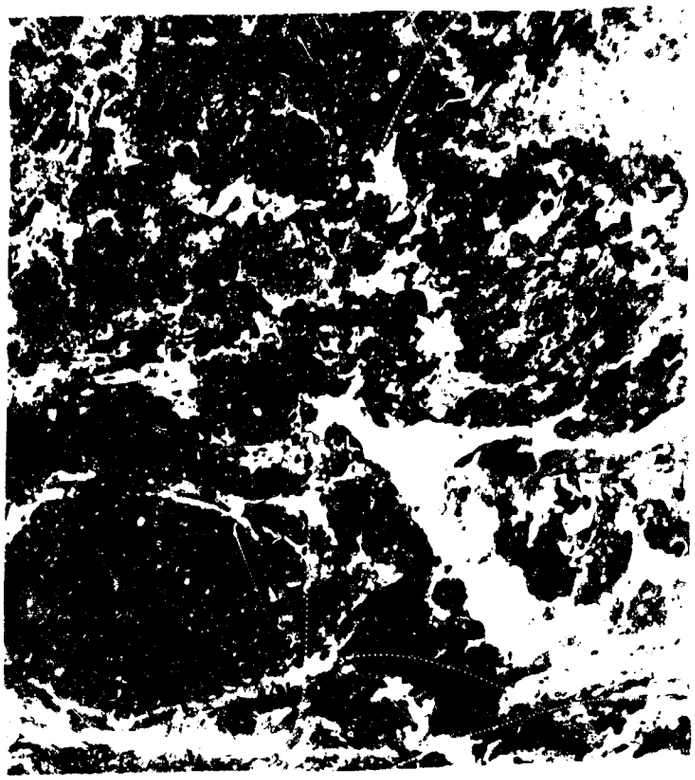


Fig. 82

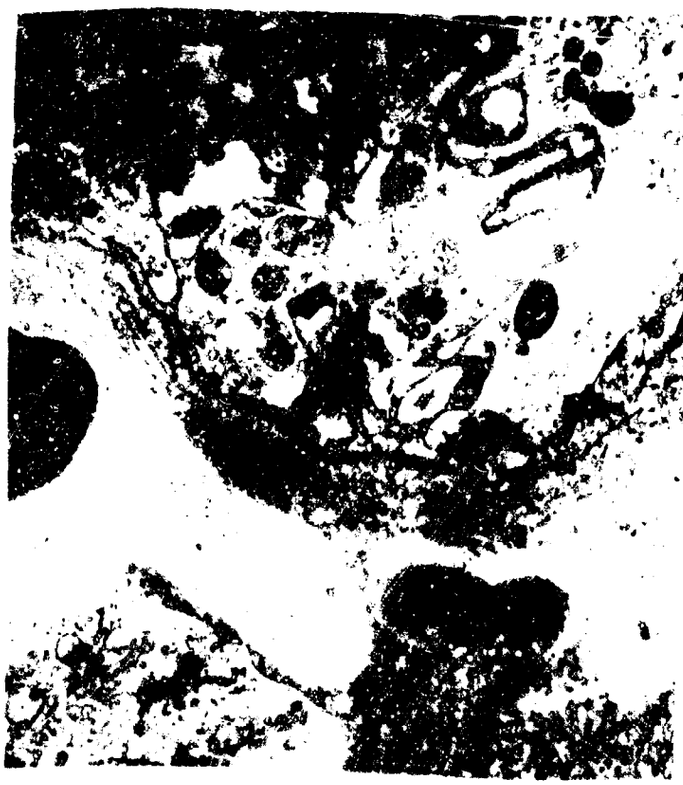


Fig. 83

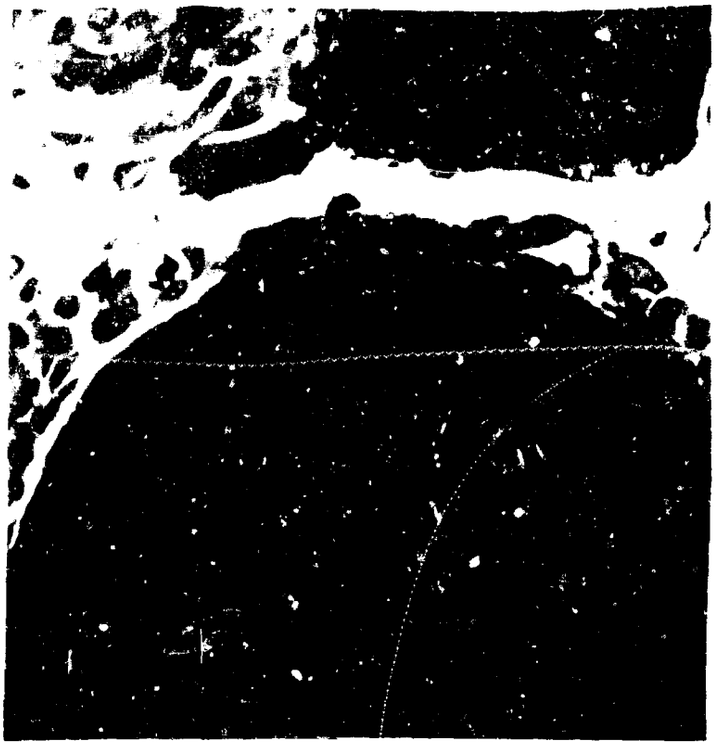


Fig. 84

Figure 85. Cells of the multilayered epithelium illustrating the electron dense nuclei with multiple nucleoli. Note the presence of the intercellular spaces between the cells. Thirteen day post injection animal. X 6,000.

Figure 86. Numerous desmosomes are present along the plasma membrane which is occasionally interupted by large intercellular spaces. Note the absence of any of the compressed vesicles within the cytoplasm of these cells. Thirteen day post injection animal. X 7,400.

Figure 87. The cells lining the basement membrane appear longer morphologically as compared to the polygonal spherical cells in other areas of the multilayered epithelium. Thirteen day post injection animal. X 6,000.

Figure 88. Higher magnification of the cells lining the basement membrane in the thirteen day animal. Note the basement membrane with its communication with the plasma membrane along with elements of rough surfaced endoplasmic reticulum. X 10,700.

Fig. 85

Fig. 86

Fig. 87

Fig. 88

Figure 89. Areas of cellular degeneration within this multilayered epithelium are illustrated. Note the whorl of agranular membranes surrounding a cytoplasmic portion of a cell, along with numerous round vesicles and a small lipid aggregation. Thirteen day post injection animal. X 6,000.

Figure 90. Evidence of cellular degeneration with alteration in organelle components is illustrated. Thirteen day post injection animal. X 10,600.

Figure 91. One micron sectioned Toluidine blue stained bladder epithelium of the fourteen day animal. Evidence of limited edema and hemorrhage along with some dysplasia of the cells is illustrated. X 640.

Figure 92. Representative area of the multilayered epithelium is illustrated. Note the numerous cellular organelles and limited evidence of intercellular spaces. Fourteen day animal. X 4,000.

Fig. 89

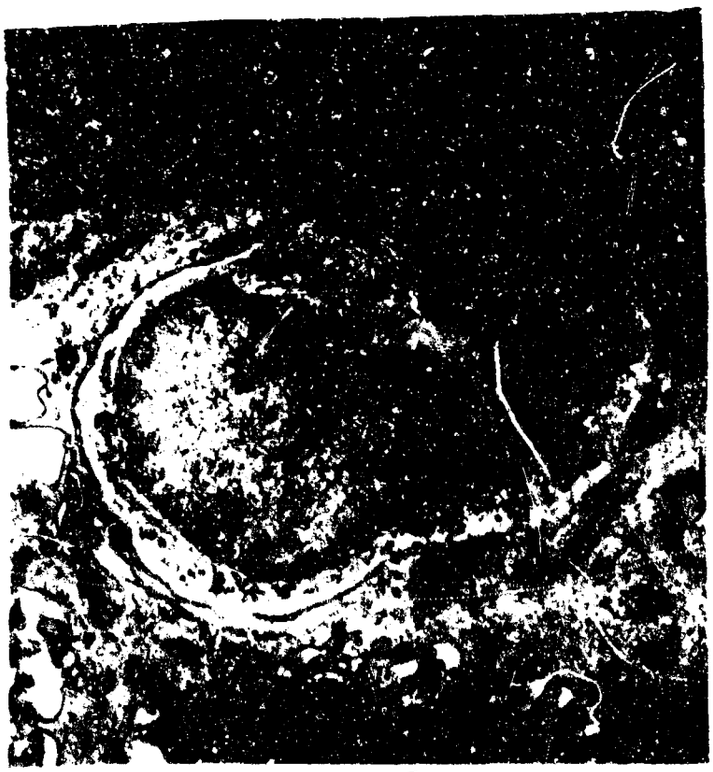


Fig. 90

Fig. 91

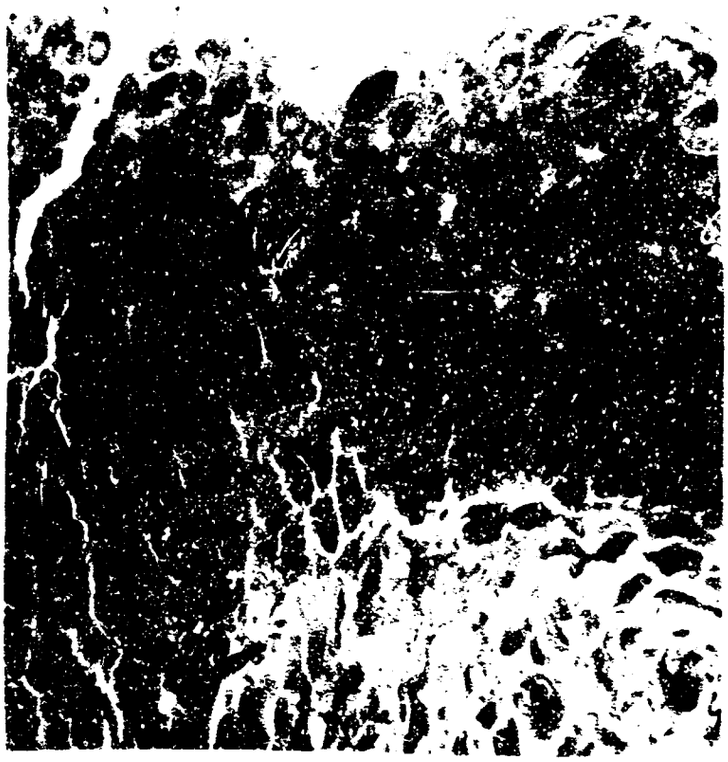


Fig. 92

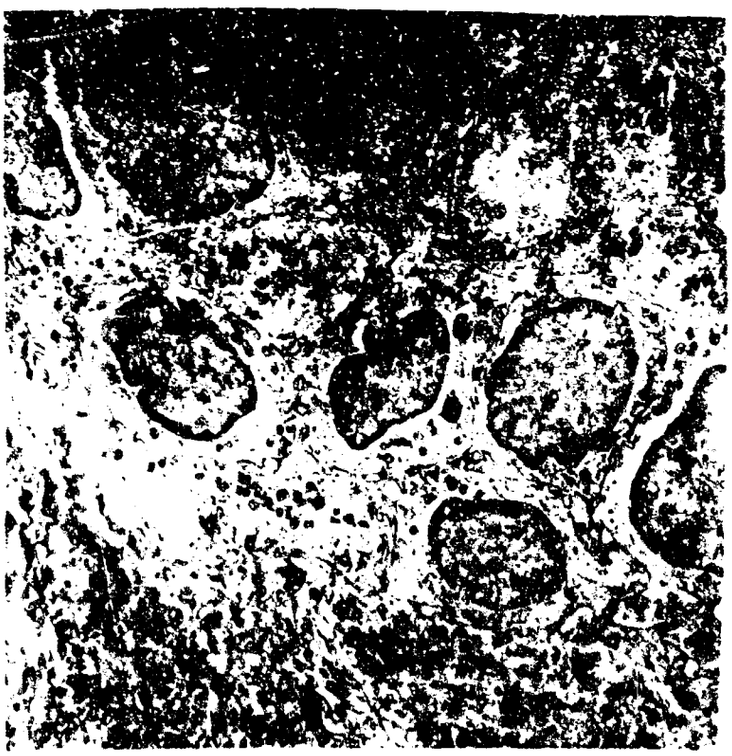


Figure 93. Superficial and intermediate cells of the fourteen day post injection animal are illustrated. Note the numerous compressed vesicles along the surface of the superficial cell. X 4,000.

Figure 94. Evidence of marked cellular degeneration in localized areas of the otherwise normal epithelial cells is illustrated. Fourteen day animal. X 7,000.

Figure 95. Evidence of the limited intercellular spaces between the cells lining the basement membrane is illustrated. Note the peripheral aggregations of nuclear chromatin and long filaments of endoplasmic reticulum. Fourteen day animal. X 5,900.

Figure 96. Between many of the cells in the fourteen day animal the plasma membrane is closely opposed with no apparent increases in the intercellular spaces. X 14,000.

Fig. 93

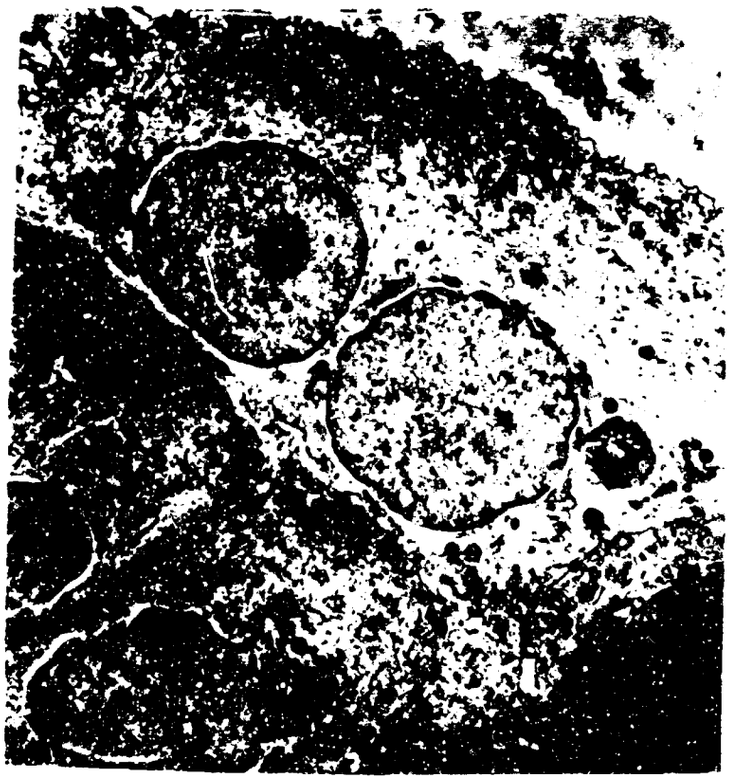


Fig. 94

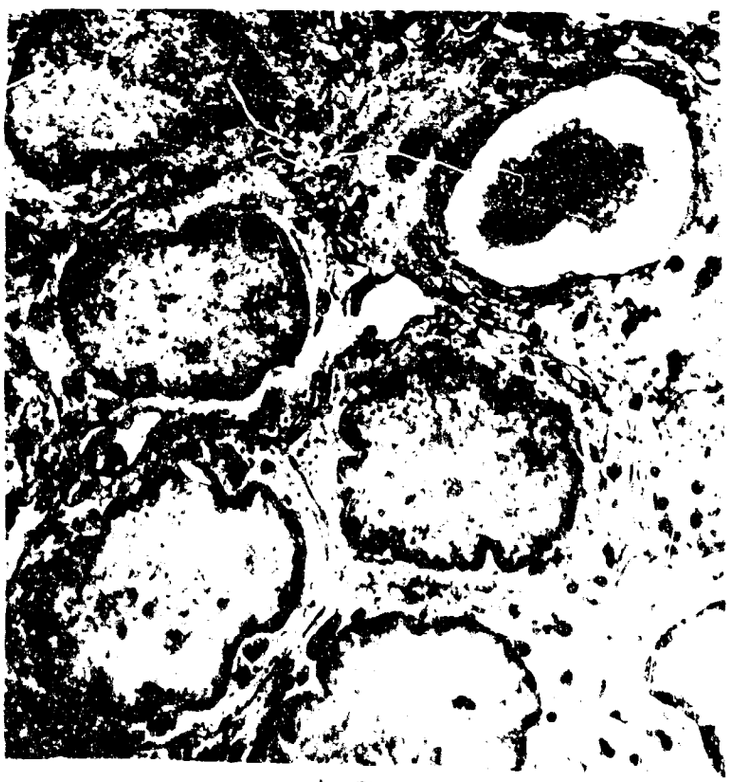


Fig. 95

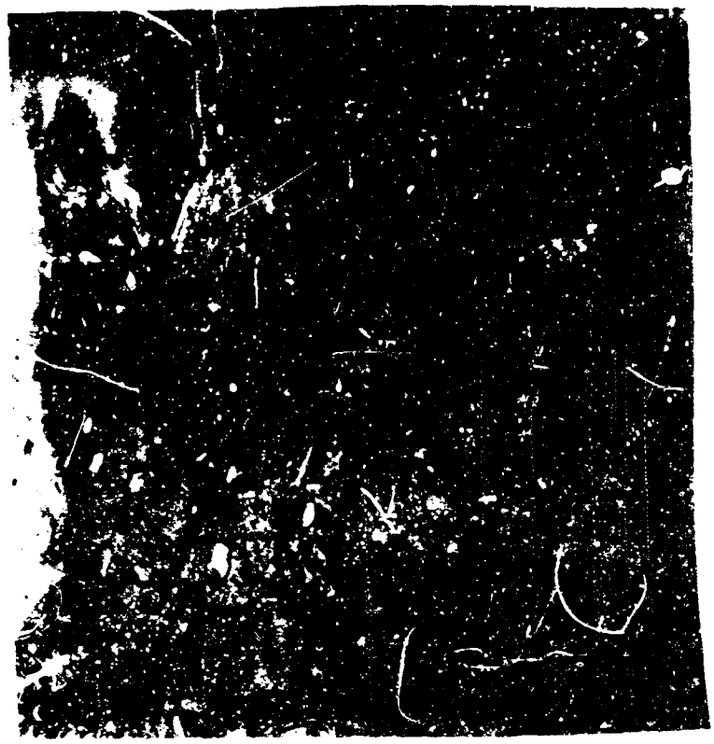


Fig. 96

Figure 97. Numerous long strands of rough surfaced endoplasmic reticulum within the cells of some of the transitional epithelial cells is illustrated. Occasional round and sometimes compressed vesicles are evident. Fourteen day animal. X 15,000.

Figure 98. Long filaments of rough surfaced endoplasmic reticulum and numerous mitochondria, uniform in size and shape and density are illustrated. Note the prominent Golgi zones within the cytoplasm of two of the cells. Fourteen day animal. X 10,600.

Figure 99. Concentric whorls of rough surfaced endoplasmic reticulum in close proximity with numerous mitochondria are illustrated. Note the fine fibrillar component within the cytoplasm is generally equally distributed. Fourteen day animal. X 20,000.

Figure 100. Area of the lamina propria with essentially normal appearing fibroblastic components. Fourteen day animal. X 4,000.

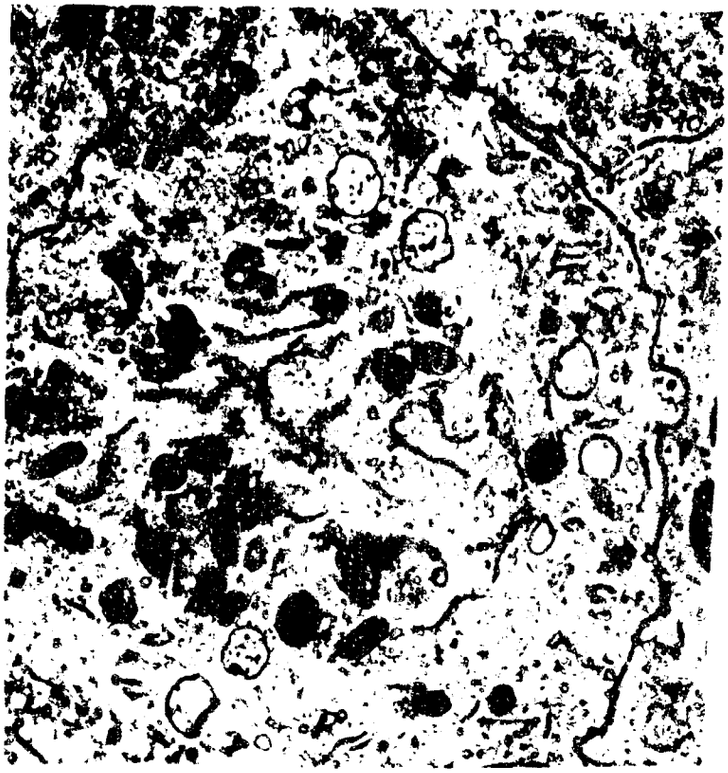


Fig. 97

Fig. 98

Fig. 99

Fig. 100

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